

**Activity Profiles & Mechanisms of Resistance of
3'-Azido-2',3'-Dideoxynucleoside Analog
Reverse Transcriptase Inhibitors of HIV-1**

by

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To investigate mechanisms of HIV-1 resistance to 3'-azidonucleoside analog reverse transcriptase inhibitors, *in vitro* selection experiments were conducted by serial passage of HIV-1_{LAI} in MT-2 cells in increasing concentrations of 3'-azido-2',3'-dideoxyguanosine (3'-azido-ddG), 3'-azido-2',3'-dideoxycytidine (3'-azido-ddC), or 3'-azido-2',3'-dideoxyadenosine (3'-azido-ddA). 3'-Azido-ddG selected for virus 5.3-fold resistant to 3'-azido-ddG. Population sequencing of the reverse transcriptase (RT) gene identified L74V, F77L, and L214F mutations in the polymerase domain and K476N and V518I mutations in the RNase H domain. Site-directed mutagenesis showed that these 5 mutations only conferred ~2.0-fold resistance. Single-genome sequencing analyses revealed a complex population of mutants that all contained L74V and L214F linked to other mutations, including ones not identified during population sequencing. Recombinant HIV-1 clones containing RT derived from single sequences exhibited 3.2- to 4.0-fold 3'-azido-ddG resistance. By contrast, 3'-azido-ddC selected for the V75I mutation in HIV-1 RT that conferred 5.9-fold resistance. We were unable to select HIV-1 resistant to 3'-azido-ddA, even at concentrations of 3'-azido-ddA that yielded high intracellular 3'-azido-ddA-5'-triphosphate levels. We have also defined the molecular mechanisms of 3'-azido-ddG resistance by performing in-depth biochemical analyses of HIV-1 RT containing mutations L74V/F77L/V106I/L214F/R277K/K476N (SGS3). The SGS3 HIV-1 RT was from a single-

genome-derived full-length RT sequence obtained from 3'-azido-ddG resistant HIV-1 selected *in vitro*. We also analyzed two additional constructs that either lacked the L74V mutation (SGS3-L74V) or the K476N mutation (SGS3-K476N). Pre-steady-state kinetic experiments revealed that the L74V mutation allows HIV-1 RT to effectively discriminate between the natural nucleotide (dGTP) and 3'-azido-ddG-triphosphate (3'-azido-ddGTP). 3'-azido-ddGTP discrimination was primarily driven by a decrease in 3'-azido-ddGTP binding affinity (K_d) and not by a decreased rate of incorporation (k_{pol}). The L74V mutation was found to severely impair RT's ability to excise the chain-terminating 3'-azido-ddG-monophosphate (3'-azido-ddGMP) moiety. However, the K476N mutation partially restored the enzyme's ability to excise 3'-azido-ddGMP on an RNA/DNA, but not on DNA/DNA, template/primer by selectively decreasing the frequency of secondary RNase H cleavage events. Taken together, these data provide strong additional evidence that the nucleoside base structure is major determinant of HIV-1 resistance to the 3'-azido-2',3'-dideoxynucleosides that can be exploited in the design of novel nucleoside RT inhibitors.

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1.0 INTRODUCTION TO THE HUMAN IMMUNODEFICIENCY VIRUS AND ANTIRETROVIRAL THERAPY

The clinical presentation of previously healthy homosexual men and intravenous drug users displaying infections of opportunistic diseases and decreased T-cell counts marked the onset of the global pandemic now known as Acquired Immunodeficiency Syndrome (AIDS) [1-3]. The etiological agent was isolated from infected individuals and independently discovered to be the human immunodeficiency virus (HIV) [4,5]. Life-long antiretroviral therapy can delay disease progression, but current drugs are limited by the development of viral drug resistance and by toxicity of the drugs on the host [6]. To date, HIV remains an incurable infection and there are no effective vaccines available. Although dozens of FDA approved antiretroviral compounds exist, a comprehensive knowledge of HIV molecular virology can lead to the rational design of novel compounds that with stronger antiviral activity, less sensitivity resistance and diminished toxicity profiles.

1.1 HUMAN IMMUNODEFICIENCY VIRUS

HIV is an enveloped, single-stranded, positive-sense, RNA virus that is a member of the lentivirus genus of the family *Retroviridae*. The replication strategy of these viruses requires reverse transcription of the RNA genome into double stranded DNA by the viral enzyme reverse

transcriptase [7,8]. Two genetically distinct types of HIV have been identified that originated in isolated primate reservoirs; HIV type 1 (HIV-1) and HIV type 2 (HIV-2). HIV-1 can be further divided into four groups, main (M), non-M non-O (N), outlier (O), and putative (P) based on the genetic differences [9-11]. Group M subtypes (A–D, F, G, H, J, and K) are spread globally, Figure 1, and originated in the chimpanzee (*Pan troglodytes troglodytes*) [12-16]. HIV-1 subtype B is responsible for most infections in industrialized nations and is often used as the model virus for research, although HIV-1 subtype C dominates most of the global infections [17]. HIV-2 subtypes are mainly restricted to West Africa and can be characterized as epidemic subtypes (A and B) and non-epidemic subtypes (C–G) [18] and originated in the sooty mangabey (*Cercocebus torquatus atys*) [19-23]. The endemic restriction of HIV-2 may be due to its lower pathogenicity over HIV-1 [24].

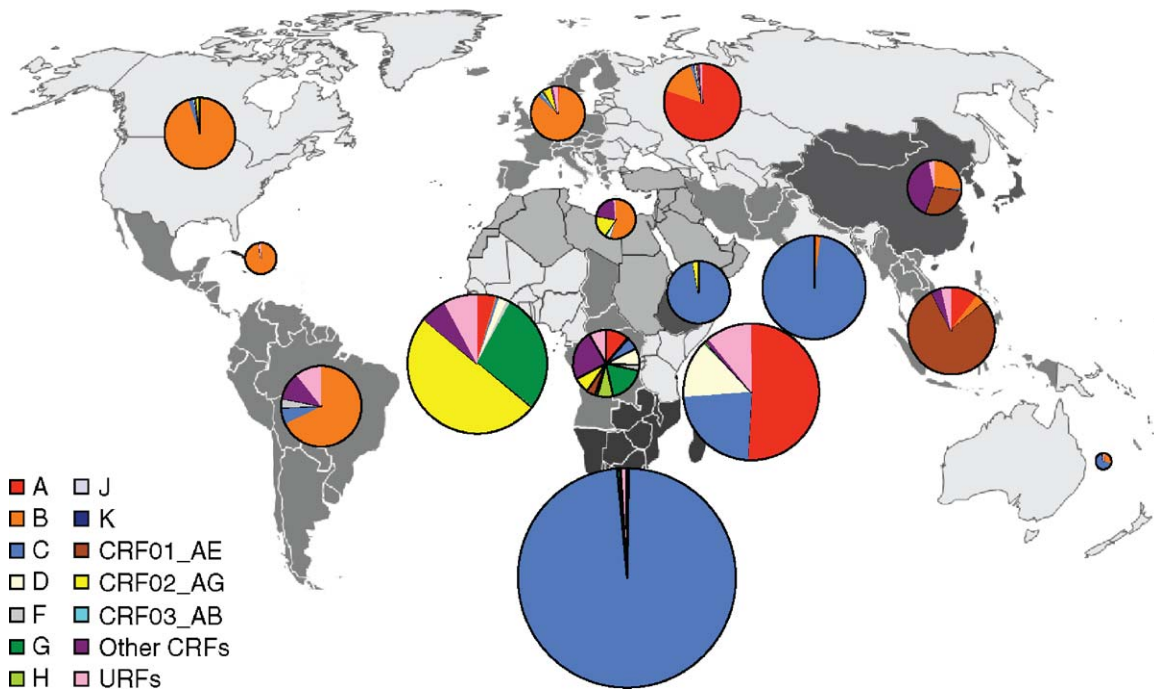


Figure 1: Global distribution of HIV-1 subtypes and recombinants

The map was divided into 15 regions consisting of groups of countries shaded in the same color. Pie charts representing the distribution of HIV-1 subtypes and recombinants in each region in 2004–2007 are superimposed on the regions. The colors representing the different HIV-1 subtypes are indicated in the legend on the left-hand side of the figure. The relative surface areas of the pie charts correspond to the relative numbers of people living with HIV in the regions CRF, circulating recombinant form; URF, unique recombinant form. Reprint with permission from Hemelaar, J., *et al.* 2011 [17].

1.1.1 Viral genome

Each individual HIV virion carries two copies of a positive-sense, single-stranded mRNA genome that are 5'-7-methylguanylate capped and 3'-polyadenylate tailed [25-27]. The complete 9.2 kb genome of HIV-1 has been sequenced and contains the classic retroviral *gag-pol-env* structural and enzymatic encoding genes, as well as additional regulatory and accessory genes, Figure 2 [28].

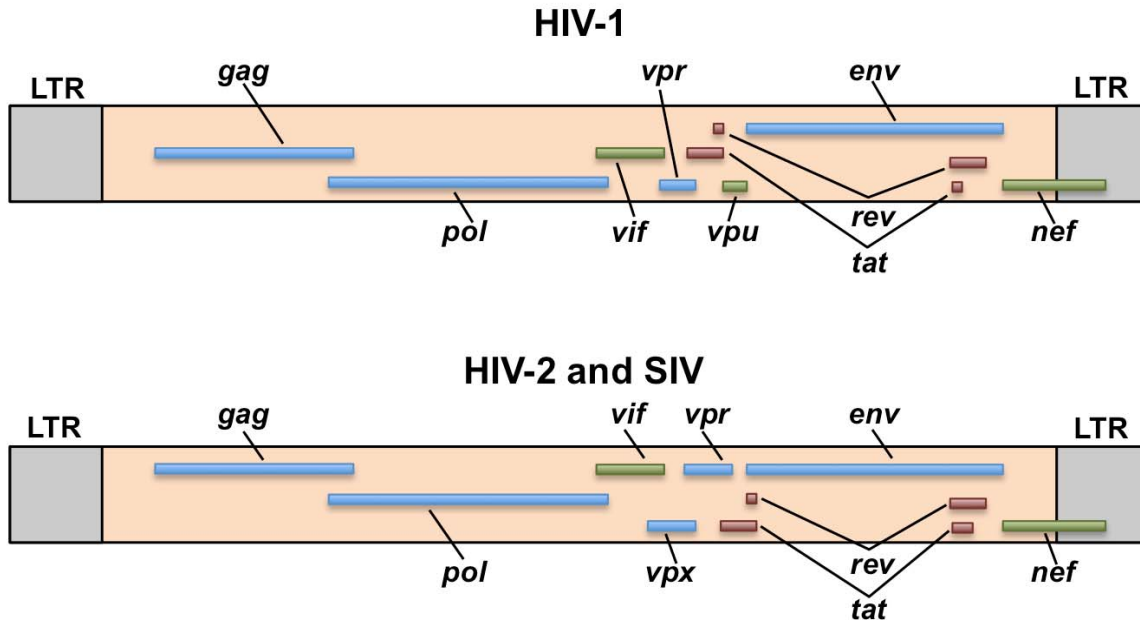


Figure 2: Genome organization of HIV-1 and SIV/HIV-2

Open reading frames that encode proteins that are efficiently incorporated into virions are in blue; those encoding regulatory proteins are in red; and those encoding other auxiliary proteins are in green. The different vertical positions denote different reading frames. The long terminal repeats (LTRs), shaded grey, contain sequences necessary for transcriptional initiation and termination, integration and binding the viral transactivator Tat.

The structural gene products are encoded collectively by *gag* and *env*. The *gag* gene is transcribed from unspliced viral mRNA into the Gag precursor protein, p55. Gag is myristoylated at the N-terminal glycine residue that is required for stable cytoplasmic membrane association and virion assembly [29]. After budding, Gag is specifically cleaved by the virally encoded protease into four proteins designated from N-terminal to C-terminal: matrix (MA, p17), capsid (CA, p24), nucleocapsid (NC, p7) and p6 [30]. After cleavage, most MA remains anchored to the inner surface of the virion membrane where it provides support to the virion structure, however a fraction of MA is incorporated into the virion core in association with integrase [31]. Before cleavage, the CA-region of Gag binds the cellular protein Cyclophilin A leading to its essential inclusion into HIV virions and, after cleavage, the CA protein forms the conical core of viral particles. [32-34]. Before cleavage, the NC-region of Gag recognizes and

binds the ψ -packaging signal of the HIV RNA genome for encapsidation into the virion [35,36]. After cleavage, NC remains in tight association the viral RNA via conserved basic residues within the viral core and upon release from the core NC stimulates reverse transcription and integration [37]. The uncleaved p6-region Gag binds the virally encoded accessory protein Vpr for encapsidation into the virion [38]. The p6-region also recruits cellular factors of the ESCRT machinery, such as Tsg101 and AIP1/ALIX, required for the efficient release of budding virions from the cell membrane [39-44]. The Env glycosylated polyprotein (gp160) of HIV-1 is expressed from a singly-spliced bicistronic mRNA and proteolytically processed by host furin protease to generate the surface (SU, gp120) and transmembrane (TM, gp41) subunits of the mature Env glycoprotein complex [45-47]. Mature Env exists as trimers on the surface and anchored in the envelope of the virion and is responsible for binding cellular CD4 receptor/CCR5 or CXCR4 coreceptor and mediating viral entry into the host cell [48,49].

The enzymatic gene products are encoded by *pol* and translated as a Gag-Pol fusion-protein precursor, p160, and are the major targets for available drugs. The *pol* gene includes the enzymes protease (PR, p11) reverse transcriptase (RT, p66/p51) and integrase (IN, p32). The expression of Gag-Pol is the result of a -1 ribosomal frame shift occurring at a UUUUUUA heptanucleotide site upstream a short stem loop near the 5'-end of *gag* [50]. Ribosomal pausing at the structured loop can allow infrequent, approximately 5 %, slippage on the poly-U tract resulting in the frame shift [51,52]. PR is a homodimer aspartyl protease and PR activity is required for cleavage of the Gag and Gag-Pol fusion-proteins described above [53-56]. RT is a heterodimer of p66 and p51, a product of PR cleavage of the C-terminal RNase H domain of p66, which has RNA-dependent and DNA-dependent DNA-polymerase activities [57-60]. During reverse transcription, RT produces a double-stranded DNA copy from the single-stranded

RNA genome. RT also has RNase H activity that degrades the RNA in a RNA/DNA hybrid duplex that allows the complementary strand of DNA to be polymerized [61]. IN is active as a dimer-of-dimers and is responsible for the biochemical reactions of 3'-processing and strand transfer leading to the stable integration of the reverse transcribed DNA proviral genome into the cellular DNA [62-64].

The regulatory gene products are encoded by *tat* and *rev*. The basal level of HIV transcriptional elongation is very low and is increased by the functions of the RNA binding protein trans-activator of transcription (Tat) [65-67]. Tat is expressed early from a fully-spliced mRNA transcript and functions by increasing the processive efficiency of RNA Polymerase II transcription [68]. In the nucleus, Tat binds to a structural element in the 5' viral mRNAs called the trans-activating response element (TAR), where it recruits the serine kinase CDK9 and other cellular co-factors [69-72]. This complex results in the phosphorylation the carboxylterminal domain (CTD) of RNA polymerase II and promotes full-length transcription of the viral genome. The regulator of virion expression (Rev) is responsible for the transition from transcription of early, fully-spliced mRNA transcripts to late, unspliced variants. Typically, unspliced mRNA is restricted to the nucleus by the nuclear pore-associated proteins, Mlp1 and Pml39, the nucleoporin Nup60, the nuclear envelope protein Esc1, and the nucleoplasmic protein Pml1 [73-76]. Rev binds a structural element found in unspliced viral mRNA called the Rev response element (RRE), inhibits splicing and escorts unspliced mRNA transcripts into the cellular cytoplasm for ribosomal translation [77-80]. This allows expression of the structural, enzymatic and accessory gene products, as well as transcription of the full-length viral genome for packaging [81,82].

The accessory gene products encoded by *nef*, *vif*, *vpr* and *vpu* are not essential for infectivity *in vitro*, but are essential pleiotropic virulence factors *in vivo*. Nef is one of the first proteins expressed following infection and has several important functions. The most prominent function is the downregulation of cell surface expression levels of proteins such as CD4, subset of major histocompatibility complex class I molecules, mature major histocompatibility complex class II molecules, CD28, CD8, CXCR4, CCR5 and the transferrin receptor [83-91]. The effects of these changes are broad and range from increased viral release to blocked superinfection and immune evasion. Vif counteracts the cytosine-deaminase, APOBEC3G, restriction factor that causes C→U transitions on the minus-strand DNA during reverse transcription when the factor is incorporated into virions [92,93]. Vif targets APOBEC3G for polyubiquitylation and subsequent proteasomal degradation to circumvent hypermutation by recruiting an ubiquitin-ligase complex including the cellular proteins elongin B and C, cullin-5 (CUL5) and ring-box-1 (RBX1) [94-98]. Vpr supports infection of dividing and non-dividing cells through effects including nuclear localization, G₂ cell cycle arrest, apoptosis, and transactivation of host and viral genes [99-110]. CD4 in the endoplasmic reticulum binds and blocks traffic of Env gp120, Vpu targets the degradation of CD4 here and restores virion production by recruiting the cullin1-Skp1 ubiquitin-ligase complex for polyubiquitylation and subsequent proteasomal degradation [111]. Vpu also counteracts the type 1 interferon induced tetherin (BST-2) from blocking virion release from the cell membrane by an unknown mechanism [112-115].

1.1.2 Viral replication

The replication cycle, Figure 3, of HIV can be outlined in an ordered sequence of events beginning with circulating virion in the blood or lymphatic systems. The initial event in viral

entry requires the recognition and high affinity binding of gp120 to the target cell receptor CD4 present on a subset of macrophages and T-lymphocytes [116,117]. CD4 recognition alone is not sufficient to induce Env-mediated entry and gp120 binding to a coreceptor is also required. Two cellular G-protein coupled receptors are important HIV coreceptors: the β -chemokine receptor CCR5 and the α -chemokine receptor CXCR4 [118,119]. Transmitted viral Env is typically able to recognize only CCR5, called R5 isolates, that is present on macrophages. However, mutations acquired during repeated replication cycles eventually convert Env coreceptor recognition to CXCR4, called X4 isolates, that is present on T-cell lines [120]. In either case, the complex formed by Env, CD4 and a coreceptor triggers a conformational change in the Env gp41 subunit resulting in membrane fusion [121,122].

The fusion of lipid bilayers releases the viral core into the cytoplasm. The core partially disassembles as CA diffuses away leading to the formation of a reverse transcription complex (RTC) containing MA, NC, RT, IN, Vpr and genomic RNA [123-125]. The conversion of viral RNA to a DNA intermediate is a defining characteristic of retroviruses and is discussed in detail below. Complete synthesis of the cDNA genome results in the formation of the pre-integration complex (PIC) containing the viral components of the RTC and additional cellular factors including, barrier to auto-integration factor (BAF), high mobility group protein HMGA, Ku and LEDGF/p75 [126-130]. The PIC is actively transported into the cell nucleus where the cDNA is stably integrated into the host genome by the activities of IN as a provirus. Here, the provirus can be transcribed or enter latency [131]. Long-lived, latently infected cells likely become the so-called viral reservoir that has made the cure for HIV infection elusive.

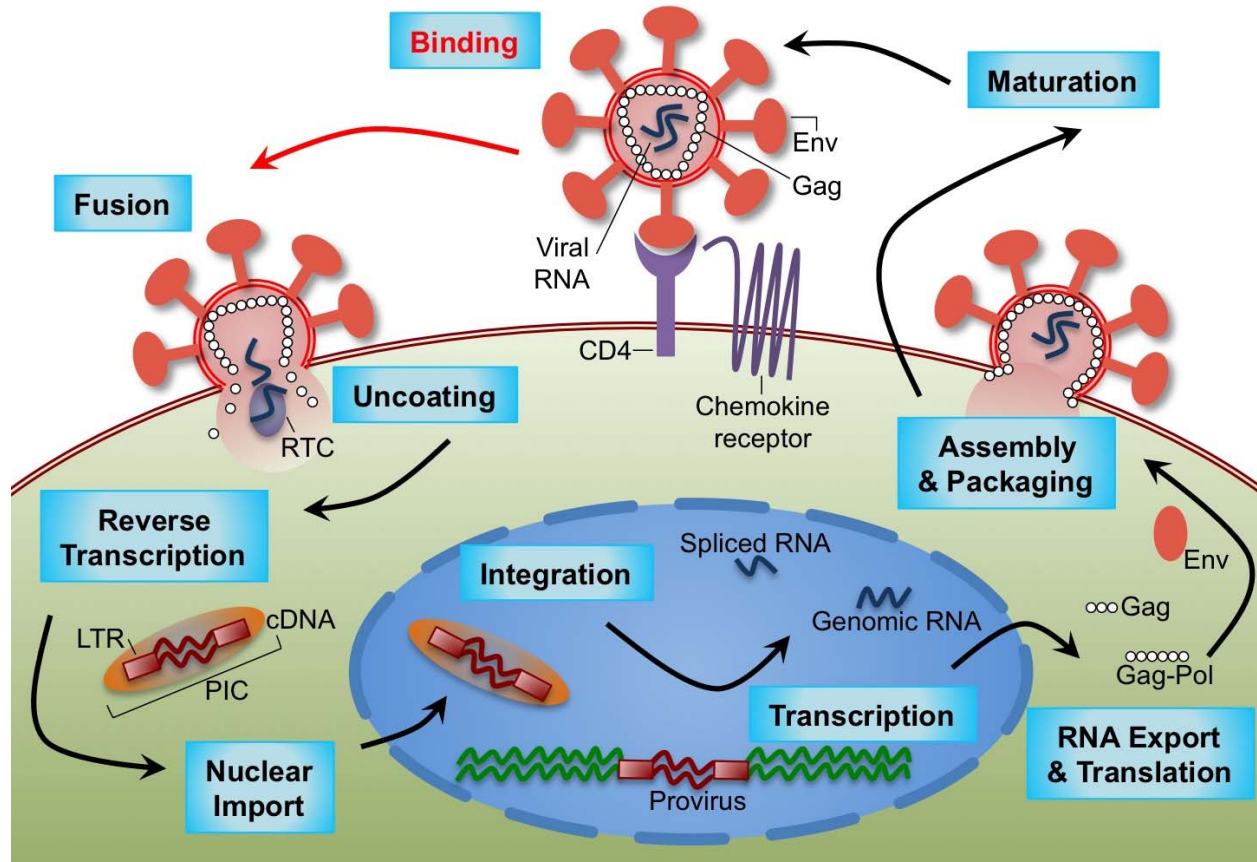


Figure 3: Replication cycle of HIV

Under favorable conditions RNA Pol II can transcribe the proviral DNA, but with poor processivity using the long terminal repeat (LTR) as the site of initiation [132,133]. Rarely, full-length transcripts are produced and fully spliced to express viral Tat, Rev and Nef. As described above, the activities of Tat greatly enhance the transcription of full-length transcripts and Rev allows the nuclear export of partially spliced transcripts for the expression of Env, Vif, Vpu and Vpr or unspliced transcripts for the expression of Gag and Gag-Pol.

The expression of the full complement of viral proteins leads to the virion assembly process. Env gp120 is synthesized in the rough endoplasmic reticulum, trafficked through the Golgi apparatus where it is glycosylated and cleaved by furin protease into gp120 and gp41 before being transported to the cell surface via the secretory pathway [134-136]. The expression

of the polyprotein Gag is sufficient for the assembly of viral like particles at the cell membrane and budding from the cell [137]. However, for infectious virion assembly, the Gag NC domain must encapsidate two copies of the viral genome, co-package some Gag-Pol and essential viral and cell factors.

Release of the immature viral particle from the cell membrane, or budding, requires the recruitment of cellular endosomal sorting complex required for transport (ESCRT) machinery [44,138-142]. These proteins interact with the Pro-rich late domains of the p6 domain of Gag and function to pinch off the viral membrane from the cell membrane. After, or during, budding, viral Pro cleaves the Gag and Gag-Pol polyproteins at nine sites to release the mature structural and enzymatic proteins. The cleavage of MA-CA triggers core assembly into the classic cone shape observed in mature virions [143-145]. The virus is released into the extracellular space in order to circulate and infect a new cell. Despite the large quantities of circulating virus often observed, many virions are non-infectious because of defects and cannot complete the necessary steps for infection.

1.2 HIV-1 REVERSE TRANSCRIPTASE

The viral enzyme RT mediates the conversion of the HIV genome during the process of reverse transcription. HIV RT is a multifunctional enzyme with RNA-dependent and DNA-dependent DNA-polymerase and RNase H activities [57-60]. This section details the structure of HIV-1 RT and the individual steps and molecular mechanisms of HIV-1 DNA synthesis.

1.2.1 Structure of HIV-1 reverse transcriptase

The HIV-1 RT gene encodes full-length p66, however the active enzyme is a heterodimer of p66 and p51, Figure 4 [146-148]. The p66 contains three domains: the polymerase (residues 1-318), connection (319-426) and RNase H (427-565). The polymerase domain is configured like that of the Klenow fragment of *Escherichia coli* DNA polymerase I in a the shape of a ‘right hand’ with fingers, palm and thumb subdomains [149]. The active site of the polymerase domain sits in the palm subdomain and contains the catalytic triad aspartate residues (D110, D185, D186) and is the site of RNA-dependent and DNA-dependent DNA polymerization. The connection domain lacks catalytic activity and the C-terminal RNase H domain is structurally similar to RNase H of *Escherichia coli* [150,151] and *Thermus thermophilus* [152] and is responsible for the digestion of the RNA component of a RNA/DNA hybrid. The p51 subunit is the product of PR C-terminal cleavage of p66 and lacks the RNase H domain [153]. The domains of p66 and p51 are folded in similar dimensions, but the spatial arrangement in each subunit varies. The p66 is described as being ‘open’ with a cleft for binding the template/primer with the 5’end of the primer sitting at the polymerase active site, while p55 is ‘closed’ and acts as a structural scaffold without catalytic activity [154].

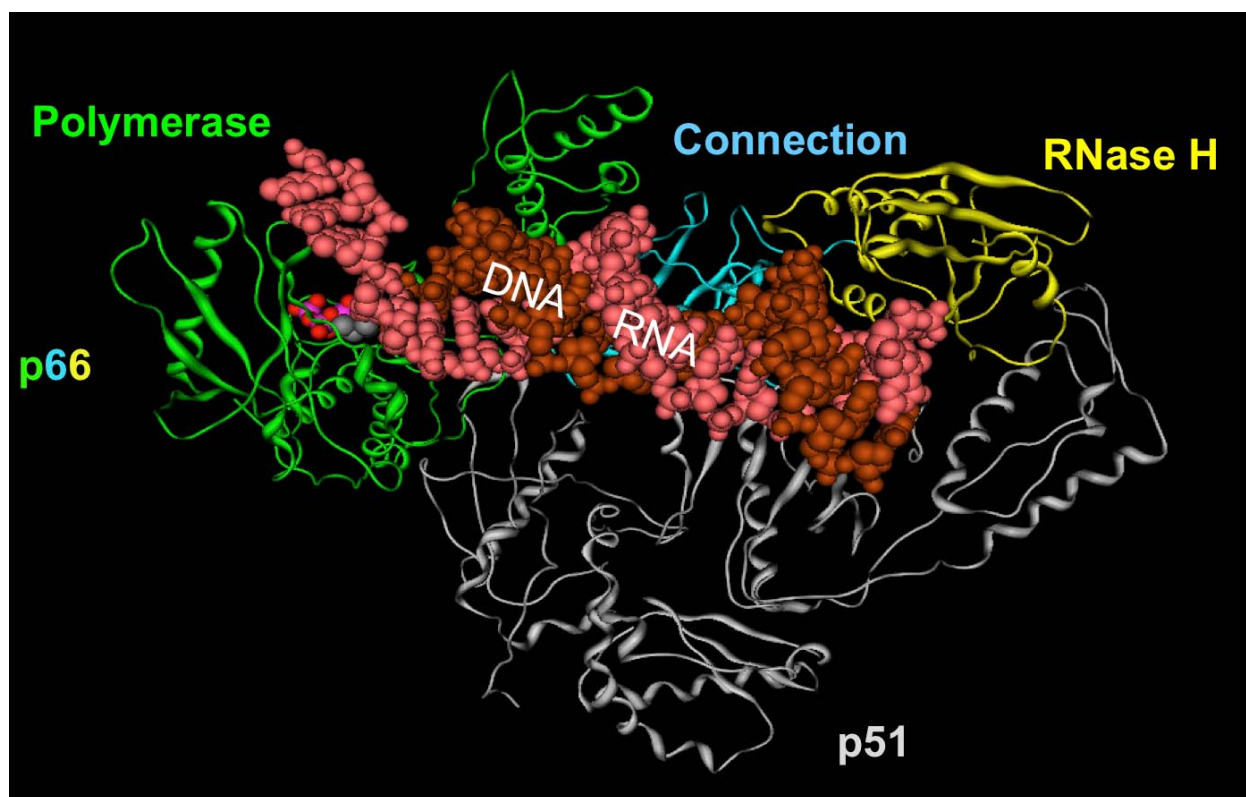


Figure 4: Structure of the p66/p51 HIV-1 RT heterodimer

The subunit p66 is colored by domain polymerase (green, residues 1-318), connection (blue, residues 319-426) and RNase H (yellow, residues 427-565), the p51 subunit is colored grey, the bound RNA/DNA Template/Primer is in light and dark red, respectively, and a bound dNTP is sitting in the polymerase active site. Rendered with coordinates PDB: 1HYS [155] using The PyMOL Molecular Graphics System, Version 1.5.0.4 Schrödinger, LLC.

1.2.2 Process of reverse transcription

The conversion of the HIV RNA genome to a DNA intermediate follows a concerted series of steps, Figure 5. The multifunctional HIV-1 RT completes all of the steps of reverse transcription, although other viral and cell cofactors are present in the reverse transcriptase complex (RTC).

1. RNA-dependent DNA-polymerization is initiated using a bound molecule of cellular tRNA^{Lys3} hybridized to the genome primer-binding site (pbs) and polymerization continues to the 5' terminus creating a RNA/DNA hybrid [28,156,157].

2. RNase H degradation of the hybrid RNA produces the minus-strand strong-stop DNA (-sssDNA) [158].
3. The -sssDNA relocates to the 3'-end of the genome using regions of homology (R) in a process called the first strand transfer [159].
4. The polymerization of the minus-strand continues using the -sssDNA as a primer.
5. RNase H degrades the genome during minus-strand synthesis, but leaves two purine-rich segments, the 3'- and central-polypurine tracts (PPT) [160]. Each RNA PPT is used as a primer for plus-strand synthesis with the minus-strand DNA and 18 nucleotides of the tRNA as a template, DNA-dependent DNA polymerization.
6. RNase H degradation of the tRNA allows the second strand transfer mediated through homology of the pbs [161]. Both segments of RNA PPT are removed by RNase H activity.
7. DNA synthesis of both the minus-strand and plus-strand from the central PPT primer continue to complete their respective LTRs. DNA synthesis of the plus-strand from the central PPT primer continues and displaces the plus-strand from the central PPT primer by approximately 100 nucleotides until the central termination signal (CTS). This displacement creates a discontinuous plus-strand with a central DNA flap that is important for viral replication [162,163].

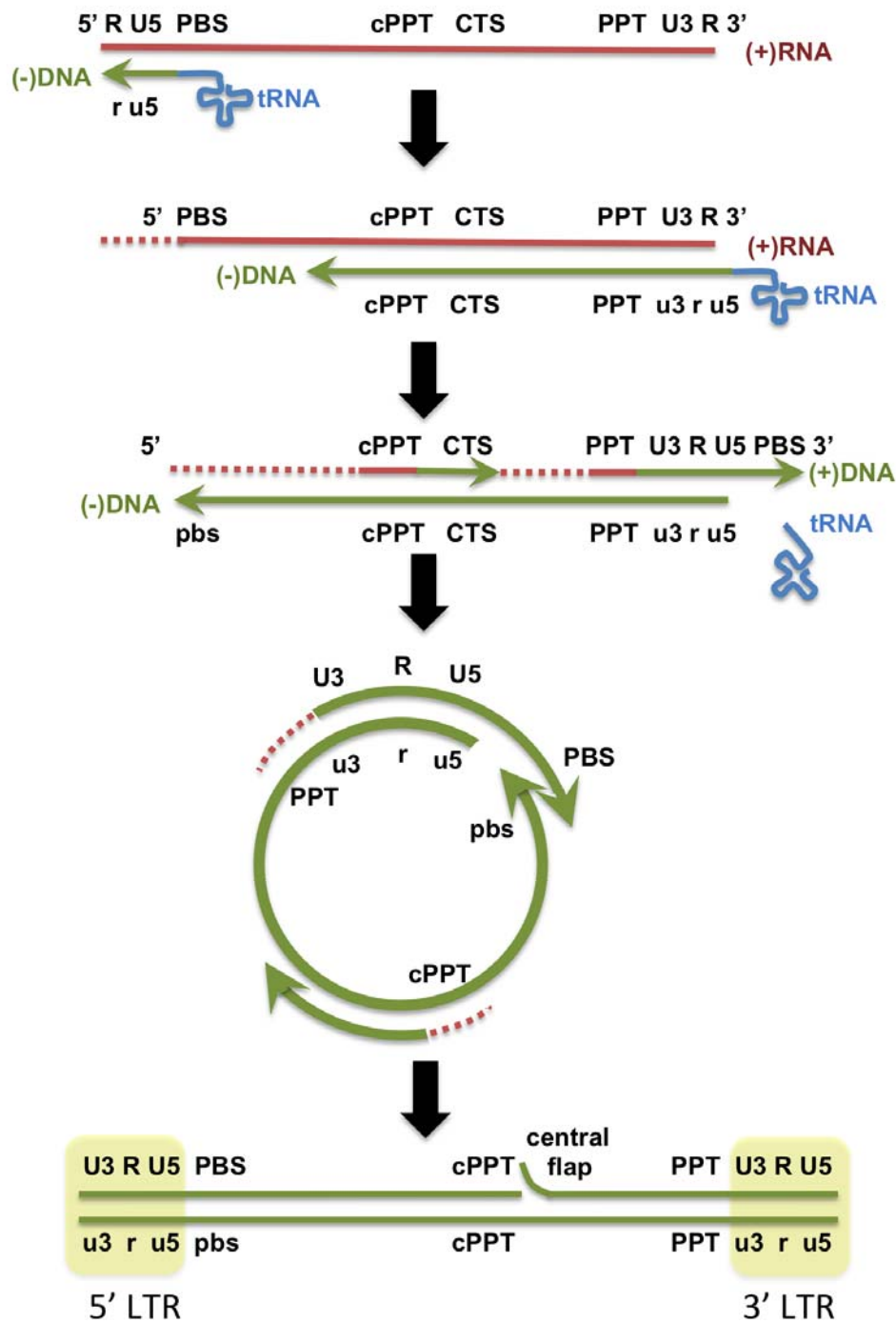


Figure 5: Reverse transcription of the HIV-1 genome

The synthesis of double-stranded HIV-1 DNA from the viral RNA genome is mediated by viral reverse transcriptase. RNA and DNA are indicated in red and green, respectively, the tRNA^{Lys3} primer is indicated in blue, RNase H degradation is indicated by dashed lines. PBS, primer binding site; cPPT, central polypurine tract; CTS, central termination signal; PPT, polypurine tract.

1.2.3 Mechanism of DNA polymerization by HIV-1 RT

HIV-1 RT is multifunctional and has both RNA-dependent and DNA-dependent DNA polymerase activities. The polymerase active site is located in the middle of the palm, fingers, and thumb subdomains of the p66 subunit. The palm subdomain positions the primer terminus in the correct orientation for the nucleophilic attack on an incoming dNTP [164]. DNA synthesis follows an ordered-sequential bi bi kinetic mechanism, Figure 6 [58,165-167]. First, RT binds the template/primer (T/P_n) at the primer-binding site (Figure 6, step 1). This binary complex is stabilized by a change of the conformation of the p66 thumb from “closed” to “open” [168,169]. Then, the dNTP binds at the nucleotide-binding site to form an $RT:T/P_n:dNTP$ ternary complex (Figure 6, step 2). Afterwards, a rate-limiting conformational change of the fingers traps the bound dNTP and aligns the 3'-OH of the primer and the α -phosphate of the dNTP to stabilize the transition state at the polymerase active site [170-172]. Then, RT catalyzes the formation of a phosphodiester bond between the primer 3'-OH and the dNMP to extend the primer strand by a single nucleotide and create a $RT:T/P_{n+1}:PP_i$ ternary complex (Figure 6, step 3). After the release of the pyrophosphate molecule, translocation of RT along the nascent elongated DNA primer frees the nucleotide-binding site for the next incoming dNTP for processive synthesis (Figure 6, step 4) or RT can dissociate from the complex and must rebind the same or different T/P for distributive synthesis to continue (Figure 6, step 5).

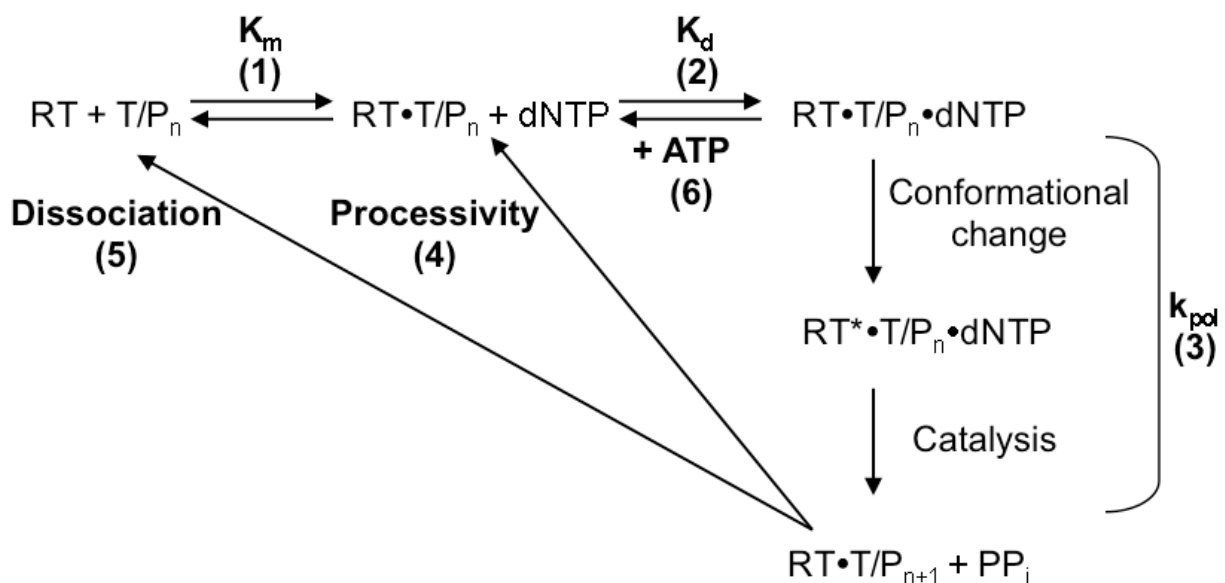


Figure 6: Kinetic mechanism of HIV-1 RT DNA synthesis

HIV-1 RT polymerization in the absence of inhibitor: (1) T/P binds to the RT enzyme; (2) dNTP binds to the nucleotide binding site of the RT•T/P complex; (3) a conformational change and catalysis must occur before (4) processive binding of the next dNTP or (5) enzyme dissociation. ATP-mediated excision is the ability of HIV-1 RT to remove the 3'-terminal nucleotide from the nascent extended primer (6).

During DNA polymerization, the conserved residues Asp110, Asp185 and Asp186 coordinate two Mg²⁺ cations, A and B, Figure 7. Cation A activates the 3'-hydroxyl group of the primer by lowering the pK_a and coordinates the nucleophilic alkoxide [173]. Both cations stabilize the hypothetical pentacovalent α-phosphorus transition state. Additionally, cation B coordinates the β- and γ-phosphates to stabilize the negative charge of the leaving pyrophosphate. This general two metal ion-catalyzed mechanism is a common feature of polymerase and nuclease enzymes across a wide range of viral, bacterial and metazoan species [173-175].

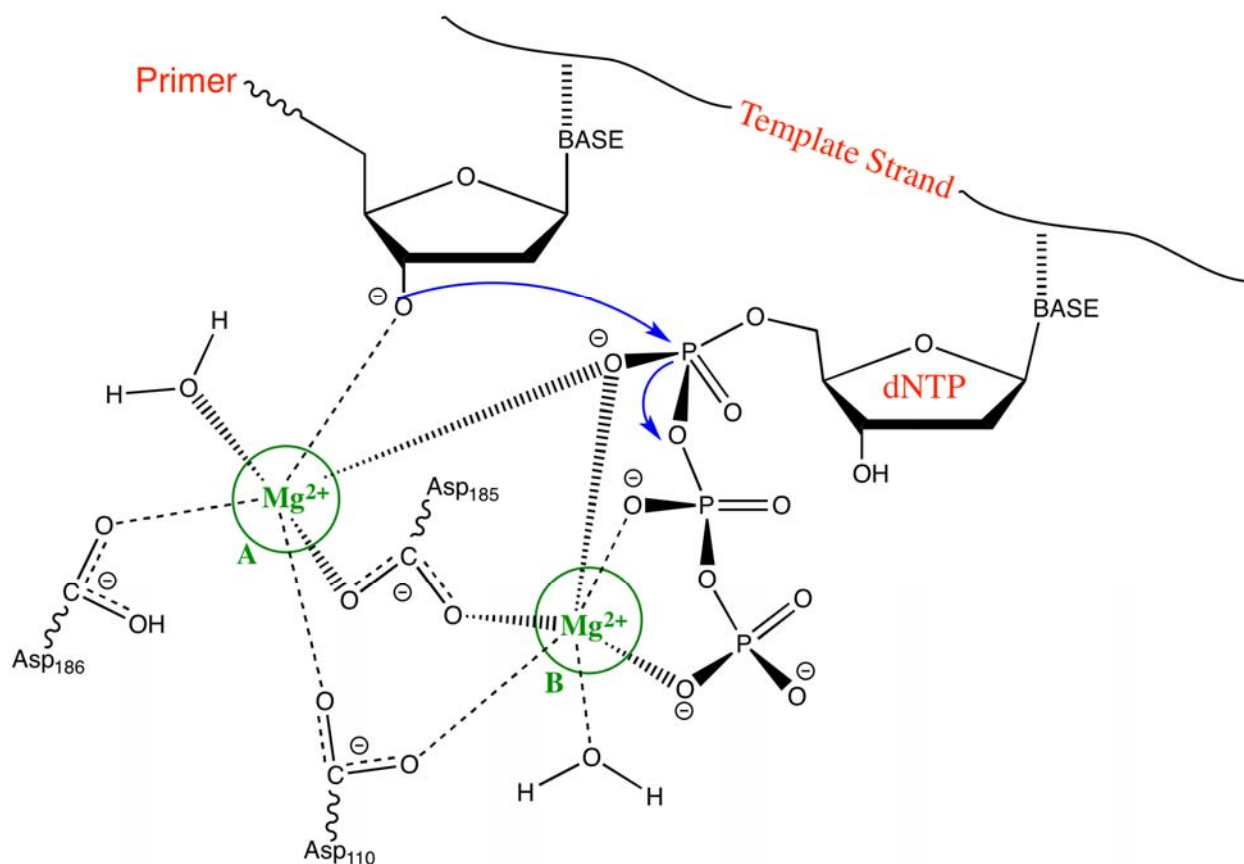


Figure 7: Molecular mechanism of catalytic DNA polymerization by HIV-1 RT

HIV-1 RT uses a two metal cation-catalyzed DNA polymerase mechanism. A conserved aspartate triad in the polymerase active site coordinates the Mg^{2+} cations, A and B. Cation A activates the primer's 3'-OH for attack on the α -phosphate of the bound dNTP. Both cations stabilize the pentacoordinate transition state. Cation B also chelates the dNTP β - and γ -phosphates and stabilizes negative charge of the leaving pyrophosphate

1.2.4 Mechanism of RNase H cleavage by HIV-1 RT

RT RNase H activity is able to selectively degrade the RNA portion of a RNA:DNA hybrid and to remove the priming tRNA and PPT during the phases of reverse transcription. The RNase H domain is located at C-terminus of the p66 subunit, 60 Å from polymerase active site or a distance of 18 nucleotides of a RNA:DNA hybrid from the RT nucleotide binding site [176]. The RNase H active site contains a conserved DDE motif containing the residues D443, E478, D498,

and D549, which coordinate two Mg^{2+} cations [177]. RNase H activity is essential and mutations in any of the D443, D498, or E478 residues abolish RNase H activity and result in replication incompetent virus [178,179]. The RNase H domain catalyzes a phosphoryl transfer by nucleophilic substitution reactions on the phosphodiester backbone of the RNA template, Figure 8. The molecular mechanism is proposed to occur through the assisted deprotonation of a water molecule (A) to produce a nucleophilic hydroxide ion that attacks the scissile phosphate group in coordination with Mg^{2+} cations [180]. The nucleophilic attack requires the simultaneous destruction of the weak P-O π -bond and results in a pentavalent transition state around the phosphorus. The transition state resolves after the reformation of the P-O π -bond and movement of electrons to the 3'-hydroxyl of the adjacent ribose sugar. This results in the cleavage of the biopolymer into two separate RNA molecules, one with a new 3'-end and one with a new 5'-end.

The specificity of RNase H cleavage for the RNA portion of the RNA:DNA hybrid is dependent on the minor groove width and its interaction with the RNase H primer grip region, a group of residues of p66 and p51 subunits that interact with the hybrid phosphate backbones [164]. The minor groove width of a RNA:DNA is intermediate between the A- and B-forms of other nucleic acids with a variable distance between 9 to 10 Å. The activity of HIV-1 RNase H is less efficient for hybrids with narrower widths, like the PPTs with a width of 7 Å due to the presence of poly-A-tracts [155,181-185]. This preserves the PPT regions to act as primers during the initiation of plus-strand synthesis during reverse transcription, but ultimately allows for their removal after the second strand transfer reaction.

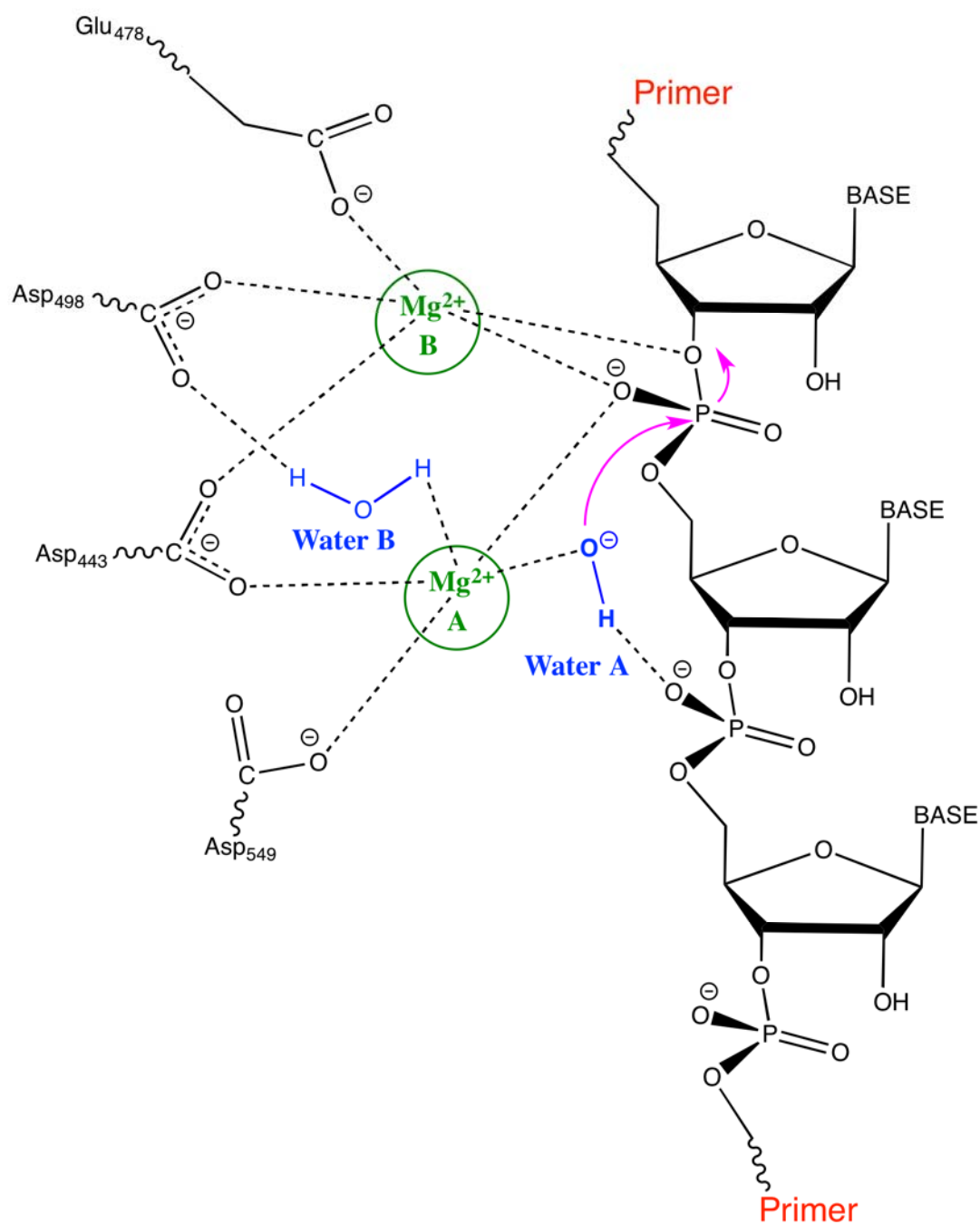


Figure 8: Molecular mechanism of catalytic RNase H activity by HIV-1 RT

HIV-1 RT RNase H activity selectively degrades the RNA portion of a RNA:DNA hybrid. In the RNase H active site of p66, water A molecule is activated by lowered pKa from coordinated Mg^{2+} A. The resulting hydroxide ion is directed to the scissile phosphate in the RNA backbone. The Mg^{2+} B stabilizes the phospholeaving group after relaxation of the pentavalent transition state through the breaking and reformation of the phosphor-carbonyl π -bond. The DNA portion of the double-stranded RNA:DNA hybrid substrate has been omitted for clarity.

RNase H catalysis can occur with three different types of cleavage modes: polymerase-dependent DNA 3'-end directed cleavage, polymerase-independent RNA 5'-end directed cleavage and internal cleavage, Figure 9. The DNA 3'-end directed cleavage acts during processive minus-strand DNA synthesis, when the RNase H cleaves the RNA in a position based on the binding of the polymerase active site to the 3'-end of the DNA primer at a rate of one cleavage per 100-200 nucleotides incorporated [155,168,172,186-188]. The RNA 5'-end directed cleavage acts during the removal of the tRNA^{Lys3} primer, PPT and cPPT tract during plus-strand DNA synthesis when the polymerase active site binds the RNA recessed 5'-end and the RNase H cleaves the RNA strand 13–19 nucleotides away from its 5'-end [185,187,189-192]. The internal cleavage is essential to remove the left over RNA during plus-strand synthesis since the RNase H cleavage is slower than DNA synthesis and any non-polymerizing RTs molecules can bind to the hybrid and degrade the RNA segment by a polymerase-independent mode [155,193].

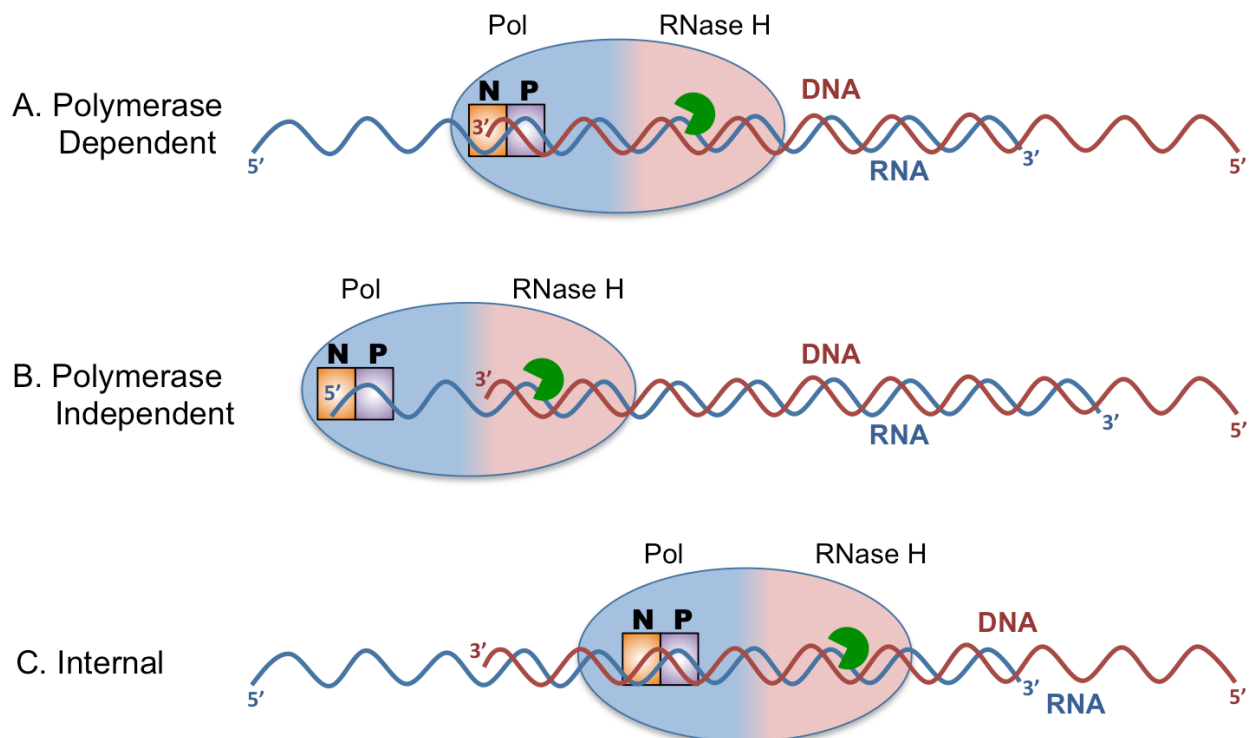


Figure 9: Modes of HIV-1 RT RNase H cleavage

1.3 ANTIRETROVIRAL THERAPY

The discovery of HIV was preceded by the clinical appearance of AIDS. HIV primarily infects CD4⁺ T cells and ultimately kills these cells during the virion replication cycle. The late stage of HIV infection results in AIDS when the depleted CD4⁺ cell count falls below 200 cells/ μ l and renders the person susceptible to opportunistic infections and malignancies that are normally controlled by a healthy immune system. The basis of antiretroviral therapy is that the protection of uninfected cells by blocking iterative cycles of viral infection permits restoration of immune function, or prevents further loss of, immune function to delay the onset of AIDS.

To date, there are 36 FDA-approved antiretroviral products, formulated either singly or in combination, to treat patients infected with HIV-1 [194]. Most are oral medicines, administered on convenient schedules in order to increase regimen adherence. Several products have been specially formulated as fixed-dose, generic-drug combinations for ease and affordability in resource-poor nations. Antiretroviral therapy can suppress viral replication for decades and dramatically increase the life expectancy of HIV-infected individuals. However, HIV is a lifelong, chronic infection with no defined cure. The therapy is compromised by non-adherence, adverse side effects and drug interactions among antiretrovirals and other medications. Each of these can lead to reduced plasma serum drug concentrations to suboptimal levels that result in the virologic failure and subsequent evolution of viral drug resistance.

1.3.1 History of antiretroviral therapy

During the first decade of the epidemic, the treatment of HIV largely consisted of prophylaxis against and management of common opportunistic infections and AIDS-related illnesses. The

first HIV-specific drug, 3'-azido-2',3'-dideoxythymidine (AZT), was discovered in the early 1990s and was administered as a single drug, monotherapy, but drug-resistance treatment failure was common [195-200]. The discovery of additional antiviral compounds in the mid-1990s allowed the standard of care to advance by including combinations of drugs to be administered together in a highly active antiretroviral therapy (HAART) [201-203]. These "cocktails" dramatically lengthened the delay of the onset of AIDS, however, early regimens consisted of complex dosing schedules, severe side-effects and heavy pill burdens with up to 20 pills/day. Improved HAART regimens were developed and continue to be enhanced to increase therapeutic efficacy and reduce the negative effects of the drugs. The most important aspect of combination therapy is the prevention of drug resistance evolution by using three antiretroviral agents directed against at least two distinct targets. Modern HAART dramatically suppresses viral replication and reduces the plasma viral load to below <50 RNA copies/mL, the limit of detection for clinical assays, and often results in a significant increase of circulating CD4⁺ T-lymphocytes [204-206].

In 2012, HIV-1 treatment guidelines for resource-rich countries recommend the initiation of HAART with three fully active antiretroviral agents for all infected adults and adolescents [6]. Early initiation of treatment reduces the risk of disease progression and transmission of HIV.

HIV drug resistance mutations have been found in both patients failing therapy and in therapy-naïve patients infected with transmitted, drug-resistant viruses. Most patients, including those with a history of failure, can be treated successfully because of the quantity of agents and distinct targets of antiretroviral drugs currently available. However, the virus continues to evolve drug resistance to current therapies, so new HIV-1 treatments will always be needed.

1.3.1.1 Reverse transcriptase inhibitors

Reverse transcription is the most targeted step for antiretrovirals approved by the FDA. There are two main classes of reverse transcriptase inhibitors called nucleoside reverse transcriptase inhibitors (NRTI) and non-nucleoside reverse transcriptase inhibitors (NNRTI). In addition, a non-nucleoside pyrophosphate analog called foscarnet also inhibits RT, but is rarely utilized because it is associated with nephrotoxicity and has limited bioavailability [207-210]. RNase H activity is another promising target for inhibition, but no drugs have been approved for this to date.

There are eight FDA-approved NRTIs currently available, that structurally resemble both purine, Figure 10A, and pyrimidine, Figure 10B, analogs of naturally occurring nucleosides. Purine nucleoside analogs include the adenine analogs 2',3'-dideoxyinosine (didanosine, ddI) and ([[(2R)-1-(6-amino-9H-purin-9-yl)propan-2-yl]oxy]methyl)phosphonic acid (tenofovir, TNV) and the guanosine analog (1S-4R)-4-[2-amino-6-(cyclopropylamino)-9H-purin-9yl]-2-cyclopentane-1-methanol (abacavir, ABC). Pyrimidine nucleoside analogs include the cytosine analogs such as 2',3'-dideoxycytidine (zalcitabine, ddC), (-)- β -L-2',3'-dideoxy-3'-thiacytidine (lamivudine, 3TC), and (-)- β -L-2',3'-dideoxy-3'-thia-5-fluorocytidine (emtricitabine, FTC) and the thymidine analogs such as 3'-azido-2',3'-dideoxythymidine (zidovudine, AZT), and 2',3'-didehydro-2',3'-dideoxythymidine (stavudine, d4T).

The general structure of all current NRTI includes the distinct lack of a 3'-hydroxyl group at the sugar moiety. While modified sugars structures are common, most active, metabolized compounds have base structures that remain identical to the natural base structures, with the exception of FTC. The HIV-1 RT still binds and utilizes these modified compounds and allows them to be effective inhibitors of the virus.

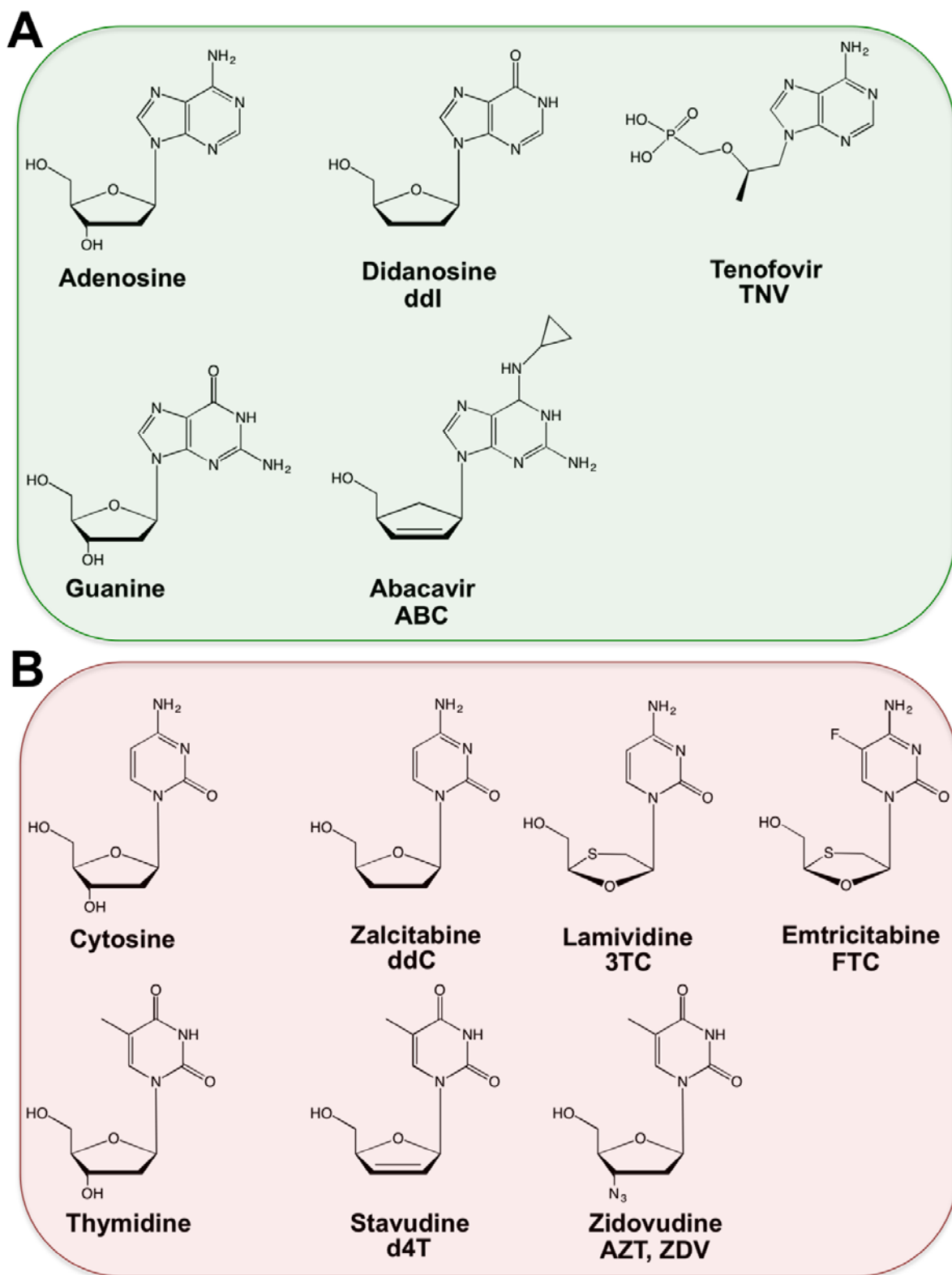


Figure 10: Structures of natural nucleoside & nucleoside analog reverse transcriptase inhibitors

Structures of all currently FDA-approved purine (A) and pyrimidine (B) analogs

All NRTI are administered as inactive parent “pro-drugs” that must be metabolized by cellular enzymes into the active compounds for the inhibition of RT, Figure 11. The compounds must be transported into cells by either passive diffusion or carrier-mediated transport after they enter the bloodstream to be metabolized [211]. A myriad of cellular phosphotransferases and nucleoside/tide kinases are required to convert the inactive drugs into deoxynucleoside-triphosphates that can then compete with natural nucleotides for binding in the N-site of RT during polymerization [212]. Incorporation of an NRTI-MP into the nascent viral DNA chain by RT results in termination of DNA synthesis because there is no 3'-hydroxyl at the primer terminus for additional nucleotides to be added, Figure 11.

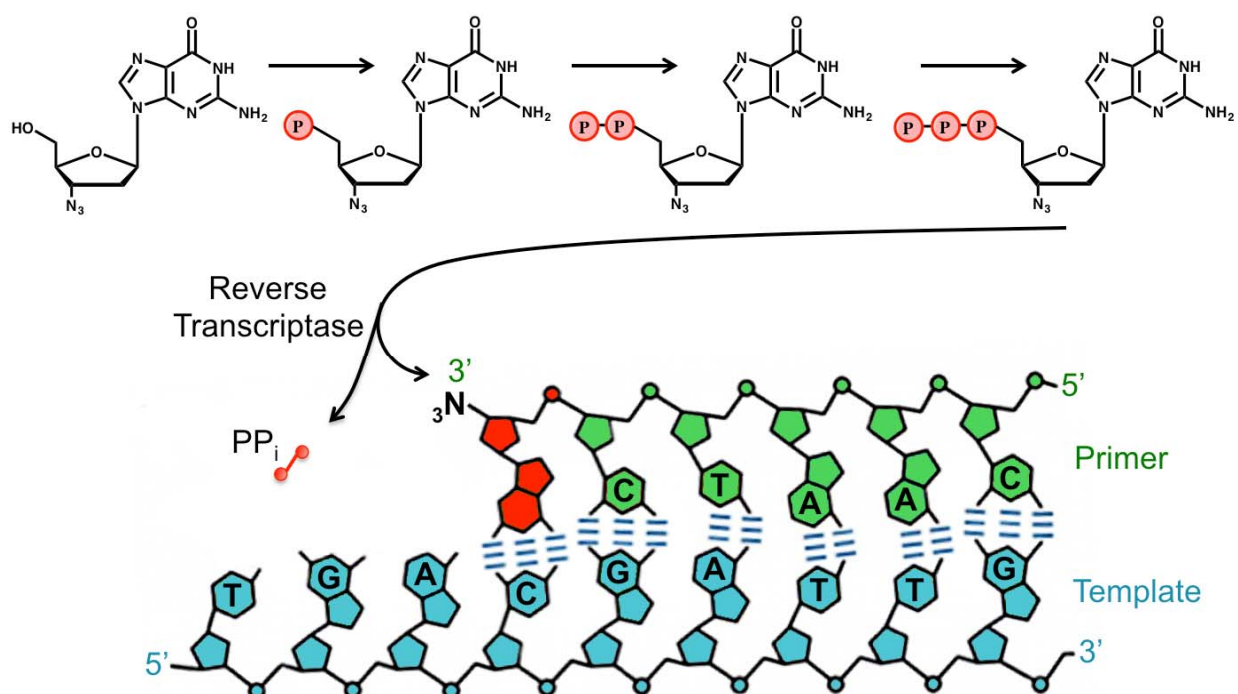


Figure 11: Intracellular phosphorylation of 3'-azido-2',3'-ddG and chain termination

The putative phosphotransferases and nucleoside/tide kinases responsible for the anabolic metabolism of 3'-azido-2',3'-ddG-triphosphate are not known, but produce the observed mono-, di- and triphosphate metabolites. Each metabolite has been identified in previous studies and the incorporation of 3'-azido-2',3'-ddG-monophosphate by RT terminates further DNA synthesis because there is no 3'-hydroxyl at the primer terminus.

1.3.1.2 Didanosine (ddI)

ddI is a 2',3'-dideoxy-structural analog of the natural purine nucleoside 2'-deoxyadenosine (dA). Cytosolic 5'-nucleotidase phosphorylates ddI to didanosine-monophosphate (ddI-MP) using inosine monophosphate or guanosine monophosphate as phosphate donors [213]. A reversible amination by adenylosuccinate synthetase and adenylosuccinate lyase converts the hypoxanthine base of ddI-MP to an adenine base to produce ddA-MP [214-216]. Additionally, 5'-adenosine monophosphate-activated protein kinase phosphorylates ddA-MP to ddA-DP and nucleotide diphosphate kinase phosphorylates ddA-DP to the active metabolite ddA-TP.

Although 2',3'-dideoxyadenosine (ddA) shows antiviral activity *in vitro*, the compound is acid labile and in clinical practice is converted by the low pH of the stomach secretions into free adenine [217]. Excess adenine is oxidized by xanthine dehydrogenase to 2,8-dihydroxyadenine, which is highly insoluble and its accumulation in the kidney can lead to crystalluria and concomitant nephrotoxicity [218]. This metabolic pathway is bypassed by the administration of acid-stable ddI instead of ddA. However, regimens including ddI are increasingly rare because it has been correlated with nucleoside analogue-associated peripheral neuropathy [219-221].

Treatment with ddI usually selects for the mutation L74V in reverse transcriptase [222]. This residue in p66 is located in the flexible finger subdomain of the β 2- β 3 loop and interacts with the template nucleotide that is base-paired to the incoming dNTP or NRTI-TP. In addition, L74 is proximal to residues Q151 and R72 that interact directly with the bound dNTP or NRTI-TP [172]. It is likely that the L74V mutation changes the packing rearrangements of the NRTI-TP to decrease NRTI-TP binding affinity or catalysis, while maintaining the ability to incorporate natural dNTP molecules. Additionally, the mutation M184V confers 2- to 5-fold ddI resistance, but only occurs in approximately ten percent of patients receiving ddI [223]. Other

mutations not selected during ddI monotherapy have also have been determined to confer resistance phenotypes. The mutation K65R causes 3- to 5-fold resistance and V75T causes about 5-fold ddI resistance *in vitro* [223-225].

1.3.1.3 Tenofovir (TNV)

TNV is an acyclic-structural nucleoside-phosphonate analog of the natural nucleotide 2'-deoxyadenosine-5'-monophosphate [226]. TNV is administered as the fumaric acid salt of the bis-isopropoxycarbonyloxymethyl ester derivative (9-[(R)-2-[[bis[[[(isopropoxycarbonyl)oxy]methoxy]phosphinyl]methoxy]propyl]adenine fumarate) of tenofovir (TDF) to increase the oral bioavailability [227]. *In vivo*, cellular carboxylesterases and phosphodiesterases quickly metabolize TDF into the nucleotide phosphonate TNV for additional phosphorylation [228,229]. The phosphonate carbon-phosphorus bond is very stable and bypasses the rate limiting initial phosphorylation towards the active drug TNV-diphosphate.

Treatment with TNV usually selects for the mutation K65R in reverse transcriptase [230-232]. This residue in p66 is located in the finger subdomain in the $\beta 3$ - $\beta 4$ loop and the ϵ -amino group of K65 interacts with the γ -phosphate of the incoming dNTP or NRTI-TP. The spatial arrangements of the mutated residue 65R may alter the binding position of the dNTP and decreases the efficiency of incorporation by approximately three-fold [233]. However, reduced tenofovir susceptibility is also observed in patients with the AZT-resistance mutations at positions M41L and L210W [234].

1.3.1.4 Abacavir (ABC)

ABC is a carbocyclic-structural analog of the natural nucleoside 2'-deoxyguanosine [235,236]. ABC is metabolized in multiple steps to produce the active inhibitor compound guanosine-

triphosphate analogue (-)-carbovir-triphosphate (CBV-TP) [237-239]. CBV is not phosphorylated, while ABC is phosphorylated by adenosine phosphotransferase to ABC-monophosphate. The 6-aminocyclopropyl group of ABC-monophosphate is removed by cytosolic deaminase to produce CBV-monophosphate. CBV-diphosphate is produced by guanydylate monophosphate kinase. Several cellular enzymes including 5'-nucleotide diphosphate kinase, pyruvate kinase, or creatine kinase can then produce the active inhibitor CBV-triphosphate.

In vitro selection experiments have identified that the mutations K65R, L74V, Y115F and M184V accumulate in RT in the presence of ABC. Furthermore, combinations of these mutations are required for ABC resistance with at least two or three mutations required to produce a 10-fold reduction in susceptibility compared to WT virus [240]. ABC monotherapy *in vivo* closely resembles the results found in cell culture [241,242]. The residues L74 and K65 are located within the finger subdomain, away from the active site and are involved with template-primer binding. L74 interacts with the incoming nucleotide through hydrogen bonding and is required for processivity and wild-type levels of replication fitness in cell culture [172,243]. The residue Y115 of p66 is in the nucleotide-binding pocket near the active site Asp185 and the neighboring M184 residue. The 4-hydroxyl group of Y155 is a steric gate that blocks the 2'-hydroxyl of ribonucleotides such that RT can only bind and incorporate dNTPs as a DNA polymerase [244,245]. The β -branched functional group of M184V can interact with the sugar structure of a bound dNTP and can sterically hinder the binding of non-natural substrates [172].

1.3.1.5 Zalcitabine (ddC)

ddC is a 2',3'-dideoxyribose-structural analog of the natural nucleoside 2'-deoxycytidine [246]. Numerous large-scale clinical trials have established that ddC-containing regimens are less

effective than other analogous combinations of NRTIs [247-251]. Furthermore, ddC is associated with two toxic effects: a dose and duration-related peripheral neuropathy and characteristic ulcerations of mucous membranes [252,253]. Therefore, ddC is largely considered as obsolete if other NRTIs are available.

The major ddC resistance mutation selected *in vivo* within RT is T69D and results in a 5-fold decrease in susceptibility [254]. Rarely, selection of K65R provides a 4- to 10-fold decrease in susceptibility, or V75T provides a 19-fold decrease in susceptibility [224,225,255].

1.3.1.6 Lamivudine (3TC)

3TC is an oxathiolane ring containing (-)-L-enantiomeric-structural analog of the natural nucleoside of 2'-deoxycytidine that was first tested as a component in a mixture of unresolved racemates of β -DL-(\pm)-2',3'-dideoxy-3'-thiacytidine (BCH-189) with an EC_{50} of 0.06 μ M in PBMC and a CC_{50} of 52.6 μ M in CEM cells [256-258]. Separation of the optical isomers by high performance HPLC showed that the β -(-) isomer was both a potent (EC_{50} of 0.002 μ M in PBMC) and non-cytotoxic (CC_{50} of >100 μ M in CEM cells), while the β -(-) isomer was both non-potent (EC_{50} of 0.2 μ M in PBMC) and cytotoxic (CC_{50} of 2.7 μ M in CEM cells) [257]. 3TC is phosphorylated to the 5'-mono-, di- and triphosphate derivatives by deoxycytidine kinase, deoxycytidine monophosphate kinase, and 5'-nucleoside diphosphate kinase, respectively [259-261].

Treatment with 3TC selects for the mutation M184V in RT p66 *in vitro* and *in vivo* [262,263]. The β -branched amino acid valine sterically hinders the binding of 3TC in the active site by interacting with the large sulfur atom of the oxathiolane ring [264,265]. Natural dNTPs can still bind in the presence of M184V and be incorporated. Interestingly, the presence M184V

makes RT more susceptible to AZT by reducing the removal of chain-terminating nucleotides by ATP-mediated phosphorolysis [266].

1.3.1.7 Emtriciabine (FTC)

FTC is the 5-fluorinated-structural analog of 3TC and is also an oxathiolane ring containing (-)-L-enantiomeric analog of the natural nucleoside 2'-deoxycytidine. The cellular metabolism of FTC follows the same path as 3TC where FTC is phosphorylated to the 5'-mono-, di- and triphosphate derivatives by deoxycytidine kinase, deoxycytidine monophosphate kinase, and 5'-nucleoside diphosphate kinase, respectively [259-261,267]. The binding affinity of FTC to RT is higher than 3TC due to the additional stabilizing hydrogen-bond formed between the 5-Fluoro group and residue R72 in RT, which may account for the increased potency and delay of selected resistance [268,269].

Resistance to FTC occurs through the RT mutation M184V via a mechanism similar to 3TC, however selected resistance was 10-fold less for FTC over 3TC under identical *in vitro* conditions [262].

1.3.1.8 Stavudine (d4T)

d4T is a 2',3'-didehydro-2',3'-dideoxy-structural analog of the natural nucleoside 2'-deoxythymidine [270]. d4T is phosphorylated to the 5'-mono-, di- and triphosphate derivatives by thymidine kinase, thymidylate kinase, and 5'-nucleoside diphosphate kinase, respectively [271-274]. In 2009, the World Health Organization recommended that all countries phase out the use of d4T because of its long-term, irreversible side effects. d4T-5'-triphosphate inhibits mitochondrial DNA polymerase γ that can lead to cumulative toxicity resulting in lipodystrophy, peripheral neuropathy, lactic acidosis, or pancreatitis [275,276].

Resistance to d4T has an interesting phenomenon in which resistance selected *in vitro* is markedly different than what has been observed *in vivo*. Cell culture studies found that K65R was selected and conferred approximately 15-fold resistance to d4T, while virus isolated from patients failing d4T regimens did not have K65R and developed the canonical AZT-resistance mutations thymidine analogue mutations (TAMs), discussed below [277,278]. Additionally, the mutation V75T has appeared in clinical isolates from patients treated with this compound and confers a 5-fold resistance over WT as well as most mutations conferring AZT-resistance [225,279].

1.3.1.9 Zidovudine (AZT, ZDV)

AZT is a 3'-azido-structural analog of the natural nucleoside 2'-deoxythymidine and was the first discovered anti-HIV compound in 1987 [195-200,280]. AZT is metabolized through the same pathway as d4T and is phosphorylated to the 5'-mono-, di- and triphosphate derivatives by thymidine kinase, thymidylate kinase, and 5'-nucleoside diphosphate kinase, respectively [272,281-283].

The most common mutations developing during AZT treatment are the well-studied TAMs. These mutations accumulate in a stepwise manner along two distinct pathways, defined as TAM1 (including mutations M41L, L210W and T215Y) and TAM2 (including mutations D67N, K70R and K219E/Q) [199,200,284,285]. High-level resistance requires the accumulation of multiple TAMs and can result in over 16,000-fold resistance [286]. At least two NRTI mutations (L74V and M184V) and two NNRTI mutations (L100I and Y181C) partially reverse AZT resistance mediated by TAMs [223,287]. The mechanism through which the 184V mutation can resensitize viruses to AZT is attributable to the finding that 184V-containing enzymes have

reduced rates of phosphorolysis, however this effect may be overcome by a further accumulation of TAMs [266].

1.3.1.10 Non-nucleoside reverse transcriptase inhibitors (NNRTIs)

NNRTIs inhibit HIV-1 RT by non-competitive binding and allosteric induction of a hydrophobic pocket near the polymerase active site [288,289]. The binding of NNRTIs causes the p66 thumb subdomain to become hyperextended because it stimulates rotations of the p66 amino acid residues Y181 and Y188 that in turn alters the conformation of the dNTP-binding site and reduces the DNA polymerase activity of RT [290]. The induced NNRTI-binding pocket consists of contains five aromatic (Y181, Y188, F227, W229 and Y232), six hydrophobic (P59, L100, V106, V179, L234 and P236) and five hydrophilic (K101, K103, S105, D132 and E224) amino acids that belong to the p66 subunit and additional two amino acids (I135 and E138) that belong to the p51 subunit. Currently there are five FDA-approved NNRTIs: efavirenz (EFV), nevirapine (NVP), delavirdine (DLV), etravirine (ETV) and rilpivirine (RPV), Figure 12.

NNRTI resistance results from amino acid substitutions in the NNRTI-binding pocket of RT that abrogate binding affinity [289]. However, K103N and Y181C are the most common NNRTI mutations selected during failing regimens [291-296]. Most NNRTI mutations generate some degree of cross-resistance with different NNRTIs, especially when multiple mutations are selected [297]. Nevertheless, the DLV resistance mutation P236L also induces hypersensitivity to other NNRTIs [298].

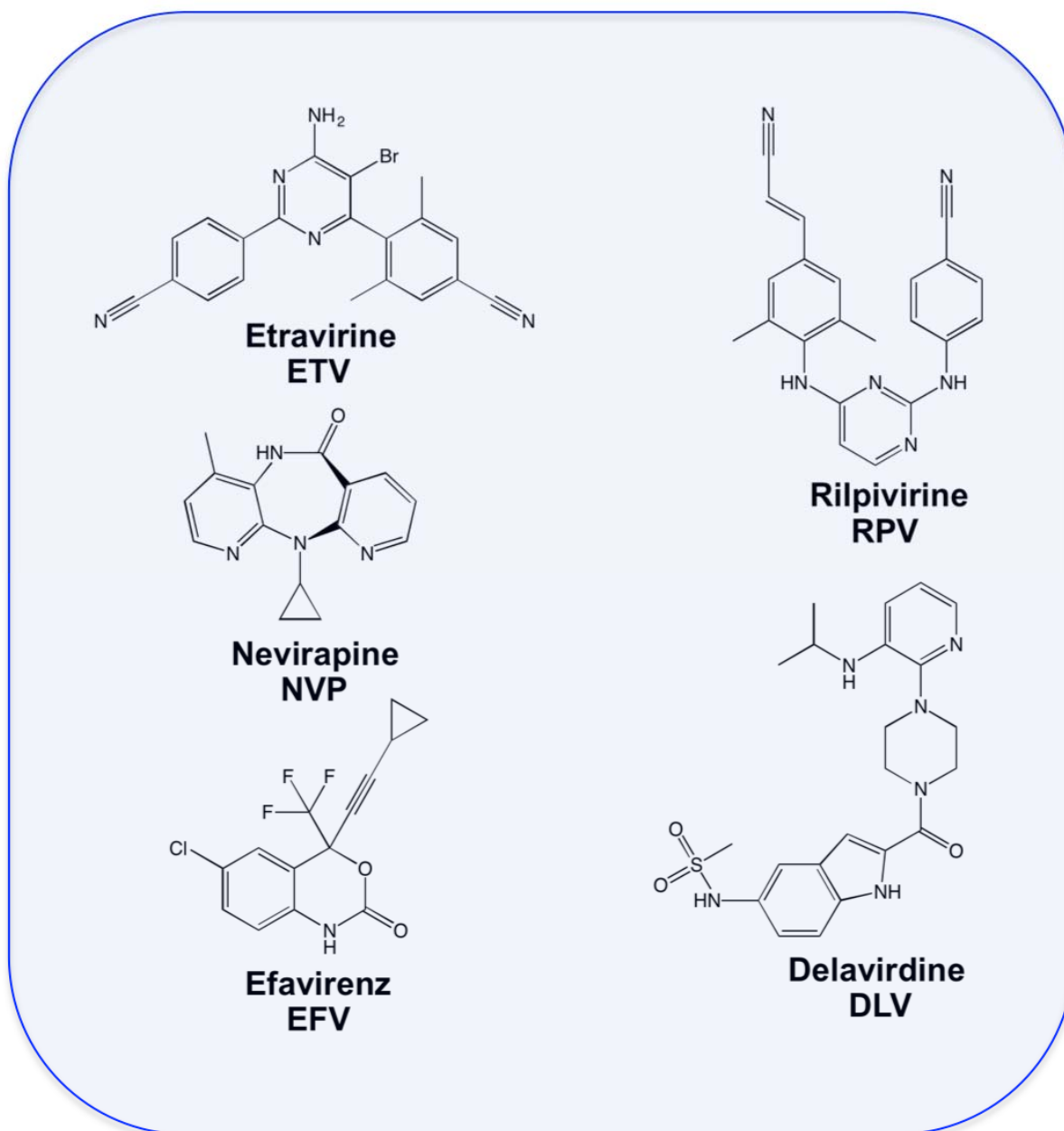


Figure 12: Structures of non-nucleoside reverse transcriptase inhibitors

All NNRTIs bind to RT in the same hydrophobic pocket, but contact different residues within the site. The first generation NNRTIs (including EFV, NVP and DLV) consist of butterfly-like structures with hydrophilic centers attached to two aromatic rings representing the wings. Second generation NNRTIs (including ETV and RPV) are diarylpyrimidines and consist of horseshoe-like structures with a central polar pyrimidine rings and two lateral hydrophobic wing-moieties.

1.3.1.11 Protease inhibitors (PIs)

The proteolytic cleavage of the gag and gag-pol polyprotein precursors into mature enzymes and structural proteins by HIV-1 PR is critical for virion maturation [53-56]. HIV PIs are compounds that competitively inhibit the action of the viral PR. These drugs prevent the maturation of HIV particles into their infectious form. Ten PIs are currently FDA-approved: saquinavir, ritonavir, indinavir, nelfinavir, amprenavir (no longer marketed), lopinavir, fosamprenavir, atazanavir, tipranavir and darunavir, Figure 13. Most PIs are prescribed with low dose of ritonavir that acts as an inhibitor of cytochrome P450-3A4 to reduce PI metabolism and extend PI serum half-life [299].

All approved PIs, with the exception of tipranavir, act as competitive peptidomimetic inhibitors that mimic the transition states of the enzyme's natural substrates. The peptidomimetic inhibitors contain a hydroxyethylene core that is an uncleavable analog of a peptide bond [300-307]. However, the non-peptidomimetic inhibitor tipranavir contains a 5,6-dihydro-4-hydroxy-2-pyrone ring as a central scaffold [308]. There have been many mutations associated with PI-resistance and cross-resistance is frequently observed between PIs due to their similar structures and mechanisms of inhibition [309]. HIV-1 develops PI-resistance through a sequential pathway by (1) selection of major resistance mutations in the PR gene, (2) acquisition of minor compensatory PR mutations and (3) development of mutations in the eight major cleavage sites of the gag and gag-pol polyprotein precursors that are better substrates for the mutant PI-resistant PR [310-312]. The major resistance mutations usually develop near the PR active site including residues involved in substrate binding. However, these changes usually coincide with a less active PR and results in decreased viral fitness that is compensated for by the accumulation of

the minor mutations over time. The mutations located within PR cleavage sites also function to compensate for the altered activities of the mutant PR.

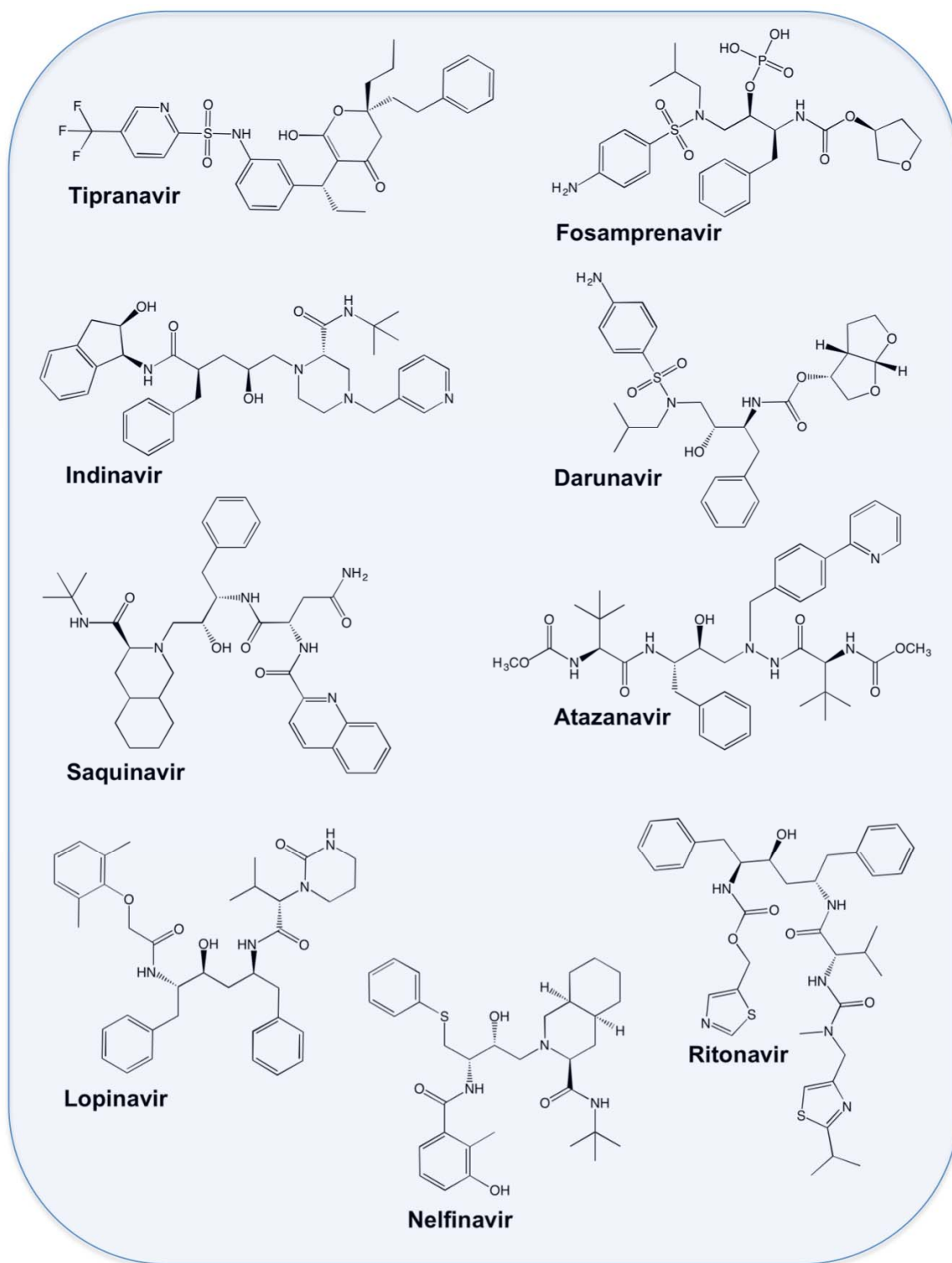


Figure 13: Structures of protease inhibitors

1.3.1.12 Integrase strand transfer inhibitors (InSTIs)

Strand transfer is a Mg^{2+} -dependent trans-esterification reaction mediated by HIV-1 IN that directs the nucleophilic attack of the two newly processed viral genome 3'-DNA ends on the backbone of the host target DNA [313]. There are two FDA-approved integrase strand transfer inhibitors (InSTIs), raltegravir (RAL) and elvitegravir (EVG), Figure 14. Both approved InSTIs sequester the IN active site Mg^{2+} ions through a chelating-triad of hydroxyl- and carboxyl functional groups and anchors into a hydrophobic pocket near the active site. The bound InSTI sterically blocks the active site from binding to target DNA and inhibits the strand transfer reaction [314,315].

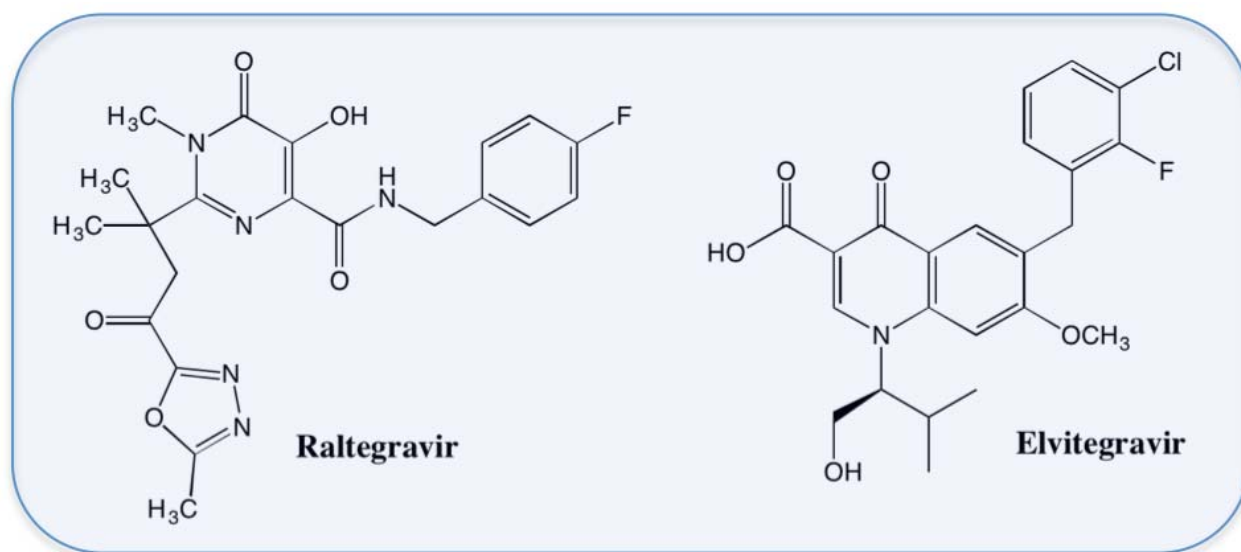


Figure 14: Structures of integrase strand transfer inhibitors

RAL resistance is clinically associated with three pathways in the IN gene, each defined by primary mutations at active-site residues Y143, Q148 or N155, while EVG resistance is only associated Q148 or N155 [316,317]. These primary mutations are correlated with secondary compensatory mutations; for Y143(C/G/R) these include T97A, L74M and E138A; for Q148(H/K/R) these include E138(A/K), G140(A/S) and Y143H; and for N155H these include

L74M and E92Q [318]. Significant cross-resistance is observed between the InSTIs irrespective of the primary/secondary mutation pathways [316,319].

1.3.1.13 Fusion inhibitors

Enfuvirtide (T-20) inhibits HIV-1 gp41 at the final stage of viral fusion with the target cell membrane [320]. T-20 is a synthetic peptide with the primary amino acid sequence CH₃CO-Tyr-Thr-Ser-Leu-Ile-His-Ser-Leu-Ile-Glu-Glu-Ser-Gln-Asn-Gln-Gln-Glu-Lys-Asn-Glu-Gln-Glu-Leu-Leu-Glu-Leu-Asp-Lys-Trp-Ala-Ser-Leu-Trp-Asn-Trp-Phe-NH₂. T-20 binds to the heptad-repeat-1 of the viral Env gp41 and blocks the conformational changes required for the insertion of the fusion peptide into the cellular membrane. Resistance to T-20 is mediated by gp41 mutations G36D, I37T, V38(A/M), N42(D/T) and N43K [321].

1.3.1.14 CCR5 antagonists

Maraviroc (MVC) is the only FDA-approved HIV-1 entry inhibitor, Figure 15 [322]. MVC binds to a hydrophobic pocket in the cellular co-receptor CCR5 that alters the conformation of the surface protein and prevents interaction with viral Env gp120 [323]. Of note, MVC is the only FDA-approved anti-HIV-1 drug that acts on a host target. Viral co-receptor tropism switching is a concern in the administration of CCR5 antagonists because infection with CXCR4-tropic virus normally leads to faster disease progression [324]. However, tropism switching has only been observed in patients with preexisting populations of X4 virus and, therefore, viral tropism must be assessed before initializing MVC containing regimens.

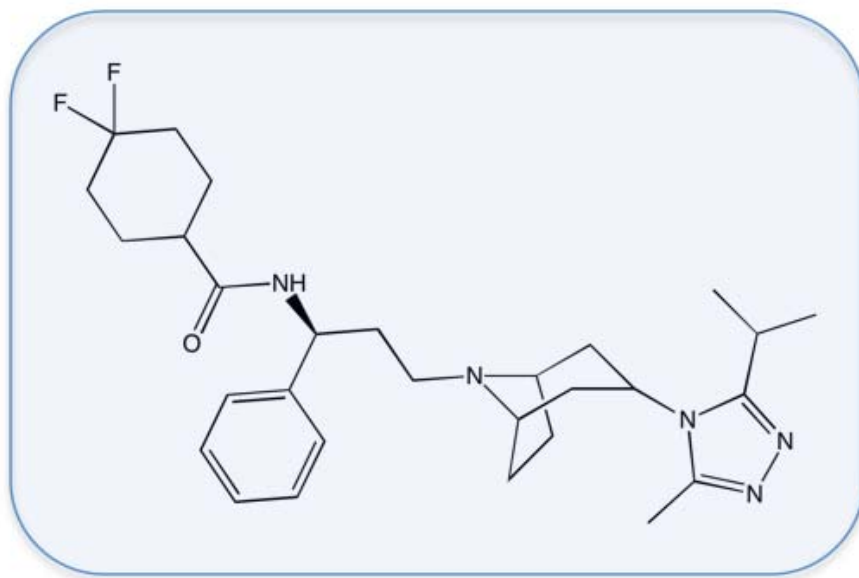


Figure 15: Structure of the CCR5 antagonist maraviroc

1.3.1.15 Recommended Antiretroviral Regimens

Novel drugs and coformulations are expanding the therapy possibilities for treatment-naïve adults infected with drug-susceptible virus. Antiretroviral therapy does not cure HIV-1 infection and requires life-long adherence for continuous suppression of viral replication to prevent emergence of resistance and viral rebound. NRTIs are largely utilized as the backbone of HIV-1 therapeutic regimens. The current recommendations of the International Antiviral Society–USA Panel for first-line therapy consists of two NRTIs (usually TDF/FTC or ABC/3TC) in combination with either 1) a NNRTI (usually EFV or NVP); 2) a ritonavir boosted PI (usually darunavir, atazanavir, or lopinavir); 3) an integrase inhibitor (either RAL or EVG) [6]. Fixed dose coformulated drugs have been developed to decrease pill burdens for NRTI including: 3TC/AZT (Combivir), ABC/3TC (Epzicom), TDF/FTC (Truvada), AZT/ABC/3TC (Trizivir) and also with multiclass combinations including: EFV/FTC/TDF (Atripla), FTC/RPV/TDF (Complera) and EVG/cobicistat/FTC/TDF (Stribald).

1.4 RT DRUG RESISTANCE MUTATIONS

HIV-1 develops resistance to NRTI by the accumulation of mutations within the gene encoding RT. The viral genome has a high rate of mutation because RT lacks proofreading activity, viral replication is rapid and large quantities of virus are produced [325]. These properties manifest in the creation of many quasi-species of HIV-1 within a host. Individual or combinations of mutations may be silent or polymorphic, result in replication deficient virus, or confer an advantageous phenotype such as drug resistance. Quasi-species that have resistance mutations will undergo natural selection during treatment and become the dominant circulating population in a patient because non-resistant virus replication will be suppressed.

The level of resistance to a specific NRTI is determined by the phenotype and number of accumulated mutations specific to each drug. This results in distinctive mutational pathways that have predictable mechanisms. For example, AZT resistance is mediated by the accumulation of TAMs (M41L/L210W/T215Y or D67N/K70R/T215F) in the polymerase domain, A371V in the connection domain and Q509L in the RNase H domain that together yield significant (934-fold) resistance [286]. The common drug resistance mutations associated with specific FDA-approved antiretrovirals are described in Table 1.

Table 1: NRTI resistance mutations in HIV-1 RT

NRTI	RT residue												
	41	65	67	69	70	74	115	184	210	215	219	371	509
ddI		K65R				L74V							
TNV		K65R			K70E								
ABC		K65R				L74V	Y115F	M184V					
ddC		K65R		T69D				M184V/I					
3TC/FTC		K65R						M184V/I					
d4T	M41L	K65R	K67N		K70R				L210W	T215Y/F	K219Q/E		
AZT	M41L		K67N		K70R				L210W	T215Y/F	K219Q/E	A371V	Q509L

1.4.1 Multi-NRTI resistance

The widespread use of HAART has favored the selection of unique patterns of mutations conferring multi-drug resistance. The four main patterns observed are K65R, the 69S Insertion Complex, the 151M Complex and accumulated TAMs. The mutation K65R confers some resistance to all FDA-approved NRTI except AZT. The 69S Insertion Complex consists of a dipeptide insertion (Ser-Ser, Ser-Gly or Ser-Ala) between codons 69 and 70 of RT together with mutations M41L, A62V, T69S, T215Y and sometimes K70R and is associated with resistance to all FDA-approved NRTI [326,327]. The 151M Complex comprises most importantly Q151M and also mutations A62V, V75I, F77L and F116Y and is associated with resistance to all FDA-approved NRTI except TNV [328]. As previously discussed, TAMs (M41L, D67N, K70R, L210W, T215Y/F and K219Q/E) are often associated with AZT-resistance, but combinations of different TAMs confer cross-resistance to all other FDA-approved NRTI as well [329].

1.5 NRTI RESISTANCE MECHANISMS

The most characterized mechanisms of NRTI resistance are NRTI discrimination that reduces the efficiency of NRTI incorporation and NRTI excision that unblocks NRTI-terminated primers [330]. These mechanisms are characterized by distinctive biochemical kinetic parameters of RT. Cross-resistance between certain NRTIs caused by similar resistance mutation pathways limits the options for therapeutic use of the compounds after a patient has previously failed a regimen. Therefore, novel NRTIs that do not select for previously described NRTI-associated mutations

are desired. However, current paradigms cannot predict the influence of NRTI structure on resistance mutations or mechanisms.

1.5.1 Discrimination

This mechanism allows mutant RT to selectively incorporate natural dNTP molecules over the NRTI-triphosphate. The catalytic efficiency is determined by two kinetic factors: the maximum rate of nucleotide incorporation (k_{pol}) and the rate of the nucleotide dissociation from the RT polymerase active site (K_d), however the effects of most selected mutations only affect one value, Table 2. These parameters are experimentally determined by pre-steady-state kinetic analysis through the use of stopped-flow rapid quench technology [165].

Table 2: Mechanisms and Resistance of Discrimination

Mutation	Mechanism	NRTI Resistance
K65R	↓ k_{pol}	ddI, TNV, ABC, ddC, 3TC, FTC, d4T
K70E	↓ k_{pol}	TNV, ABC, 3TC
L74V	↓ k_{pol}	ddI, ABC, ddC
^aQ151M	↓ k_{pol}	ddI, ABC, ddC, 3TC, FTC, d4T, AZT
M184I/V	↑ K_d	ddI, ABC, 3TC, FTC

^a Q151M Complex contains Q151M and also mutations A62V, V75I, F77L and F116Y.

1.5.2 Excision

This mechanism allows mutant RT to catalyze ATP- or pyrophosphate-mediated removal of an incorporated NRTI-MP from a chain-terminated primer. Primer unblocking restores the 3'-hydroxyl group of the -1 nucleotide of the primer strand and allows RT to resume polymerization

until another NRTI-MP is added. Only TAMs and the 69S Insertion Complex have been associated with excision activity.

The terminating NRTI-MP must be located in the N-site of the RT polymerase active site for the excision reaction to occur. Binding of the next correct nucleotide can prevent excision by translocating the primer-NRTI-MP into the P-site. This forms a dead-end complex because RT is unable to polymerase as a result of the incorporated NRTI-MP and is unable to excise the compound due to its position in the P-site [331]. Biochemical analyses provide evidence for several mechanisms for increased excision in RT with TAMs, including binding ATP in an orientation that positions the γ -phosphate for nucleophilic attack, faster rate of excision of the terminator and a shift in the equilibrium favoring the terminating NRTI-MP in the N-site over the P-site [332-335].

Although AZT-MP is the most efficient FDA-approved substrate for excision, other terminating NRTI-MP can be removed as well. Different factors impact the ability of a the NRTI-MP to be excised, such as the base and sugar structures of the active nucleotide analog [336]. The 3'-azido group of AZT-MP is not the primary determining factor for excision because 3'-azido-2',3'-ddC, and 3'-azido-2',3'-ddU are also efficiently removed from the end of a terminated primer by TAM containing RT, while the excision rates for 3'-azido-2'-3'-ddA and 3'-azido-2',3'-ddG are significantly reduced [337]. Current evidence suggests that the pyrimidine-based inhibitors are better substrates than their purine-based analogs. The sequence and type of template (RNA or DNA) can also influence the rates of excision. Rescue of DNA synthesis happens at similar rates on primers annealed to either DNA or RNA templates, however the amount of terminator removed from a RNA-bound primer is less than a DNA-bound primer of identical sequence. This suggests that the rate of excision is faster in the context of a

DNA primer. Additionally, excision by TAM containing RT is not efficient during the initiation of minus-strand DNA synthesis, providing evidence that NRTI-mediated inhibition varies during different stages of the reverse transcription [338,339].

The RNase H activity of RT has also been implicated to influence excision rates of a terminated primer bound to an RNA template. Two mechanisms are possible for the enhanced excision by decreased RNase H activity, including decreased template switching during reverse transcription and template preservation to provide an increased amount of time available for excision. This is supported by the observations that mutations in the RNase H primer-grip region or connection subdomain increase AZT-resistance and accelerated excision by bound EFV decrease AZT-resistance in TAM containing RT [340-343].

1.6 IMPORTANCE OF NRTI DISCOVERY

NRTI drug resistance can severely limit the therapeutic options for an HIV-1 infected individual, so new analog with favorable cross-resistance profiles are constantly desired. NRTI have been historically discovered by the initial synthesis of novel compounds, followed by the empirical testing for *in vitro* cytotoxicity and antiretroviral activity against wild-type or drug-resistant virus. This is a cumbersome process and has the potential to miss highly active compounds because the structural determinants of activity may not be known. Our group has previously conducted numerous studies to systematically determine various contributions of both the sugar structure and base towards an individual NRTI's anti-HIV-1 activity. This knowledge has contributed to the rational design of novel analogs with activity against virus with TAM containing RT.

1.6.1 Structurally diverse analogs

Our group has previously completed a systematic evaluation of a series of related, but structurally diverse, NRTI to identify the structural components of analogs that confer anti-HIV-1 activity *in vitro* [336]. It was found that both the sugar and base moieties influenced the antiviral activities in a predictable manner in respect to RT with the multi-NRTI resistance mutation K65R. The most potent sugar structure for the inhibition of this resistant virus was 3'-azido-2',3'-dideoxyribose that showed little to no resistance over wild-type virus. All other analogs tested (2',3'-dideoxy-, 2',3'-didehydro-, 2'-fluoro-2',3'-didehydro-, 3'-thia-2',3'-dideoxy-, L-3'-thia-2',3'-dideoxy-, D- or L-dioxolane- ribose) showed levels of resistance ranging from 2.3- to 77-fold over wild-type virus. Additionally, the base structure was identified to be an important factor for activity. For the 3'-azido-2',3'-dideoxy series of analogs, the order of activity by EC₅₀ values was T > A > G > C (ranging from 0.20 to 15.0 μ M), and the order of resistance by K65R virus was C > G > T > A (ranging from 2.5- to 0.9-fold resistance over wild-type). However, it was not known if these analogs would retain activity against a virus containing the AZT-resistant TAMs.

1.6.2 Importance of 3'-azido-2',3'-dideoxypurine analogs

Our group has previously performed in depth biochemical analysis of the 3'-azido-2',3'-dideoxy series of analogs to determine the kinetics of incorporation and excision, as well as to determine the *in vitro* inhibition of virus with AZT-resistance mutations. Purified wild-type or TAM containing RT (D67N/K70R/T215F/K219Q) was used to determine the ability to incorporate and then excise each analog, Figure 16. It was confirmed that TAM containing RT is more efficient

than the wild-type RT in this regard for AZT, as expected, as well as 3'-azido-2',3'-dideoxycytidine (3'-azido-ddC), but not either 3'-azido-2',3'-dideoxyguanosine (3'-azido-ddG), or 3'-azido-2',3'-dideoxyadenosine (3'-azido-ddA) [337].

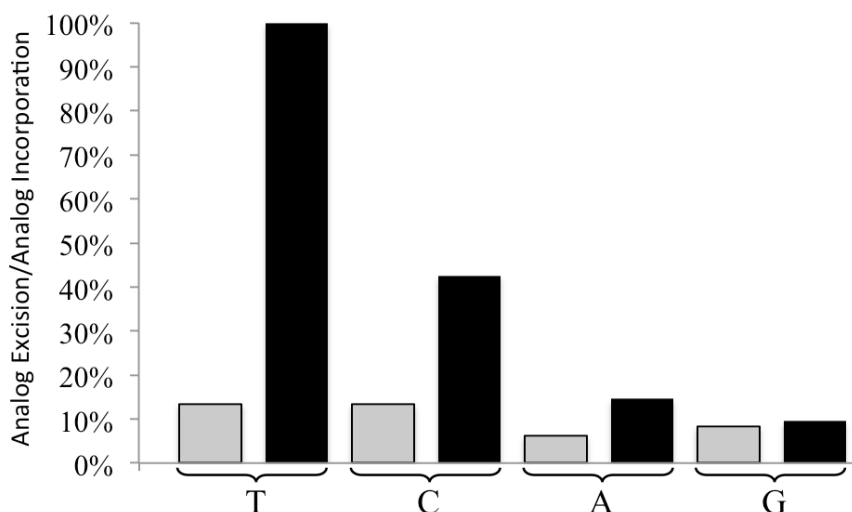


Figure 16: Relative efficiency of excision to incorporation of 3'-azido-ddNucleotides

Each 3'-azido-ddNucleotide (T, Thymidine; C, Cytidine; A, Adenosine; G, Guanosine) was evaluated *in vitro* for excision and incorporation activity by wild-type (*grey bars*) or TAM containing (*black bars*) purified recombinant RT. The individual values for the catalytic efficiency of excision and incorporation were previously determined [337].

The HIV-1 susceptibility of each analog was tested *in vitro* with virus containing wild-type or TAM containing RT. The results correlated well with the biochemical data in that the TAMs conferred resistance to both AZT (11.7-fold over wild-type) and 3'-azido-ddC (10.7-fold over wild-type), but not either 3'-azido-ddG (1.3-fold over wild-type) or 3'-azido-ddA (1.2-fold over wild-type), Table 3 [337].

Table 3: Antiviral activity of 3'-azido-ddN analogs

Base	^a EC ₅₀ , μ M		
	HIV _{WT}	^b HIV _{TAMs}	^c Fold Resistance
T	0.031 \pm 0.02	0.36 \pm 0.2	11.7
C	15.6 \pm 10.7	160.8 \pm 24.1	10.7
G	7.09 \pm 4.63	10.3 \pm 8.3	<u>1.3</u>
A	7.9 \pm 3.4	8.6 \pm 5.4	<u>1.1</u>

^a data are the mean \pm standard deviation of three independent experiments determined in P4/R5 cells [337].

^b HIV_{TAMs} is HIV-1 with RT mutations at D67N/K70R/T215F/K219Q.

^c Fold resistance is determined as EC₅₀ HIV_{TAMs}/ EC₅₀ HIV_{WT}.

Further studies show that 3'-azido-ddG and 3'-azido-ddA have anti-HIV-1 activity in a variety of cell types including P4/R5 (HeLa-based reporter cell line), MT-2 (HTLV-1 infected human T cell line) and primary human peripheral blood mononuclear cells (PBMC) with EC₅₀ values ranging from approximately 0.2 to 10 μ M [344]. The activities of these compounds were also evaluated against a panel of viruses harboring a variety of common NRTI-resistance mutations, Table 4 [344]. It was observed that the 3'-azido-2',3'-dideoxypurine analogs are potent inhibitors of these viruses, though there was some resistance associated with the 69S Insertion and Q151M Complexes. Although 3'-azido-ddA appears to retain slightly more resistance against known drug resistance mutations, 3'-azido-ddG has additional favorable properties that make it desirable. There was low cytotoxicity of 3'-azido-2',3'-ddG (CC₅₀ range of >100 to >270 μ M) across five different types of cells including PBMC, MT-2, P4/R5, Vero (*C. sabaues* epithelial kidney cell line), CEM (Human T cell lymphoblast-like cell line) and HepG2 (human hepatocellular carcinoma cell line). Additionally, there was a linear dose-response for the cellular uptake of 3'-azido-2',3'-ddG and metabolism to the active triphosphate compound with a intracellular half-life of approximately 9 hours. The cytotoxicity of 3'-azido-

ddA was more substantial with CC₅₀ values of 74.3 μ M (PBMC) and 50.6 μ M (both Vero and CEM cell lines). Additionally, 3'-azido-ddG did not show signs of mitochondrial toxicity, while 3'-azido-ddA decreased the physical amount of mtDNA and increased lactic acid production [344].

Table 4: Antiviral activity of 3'-azido-2',3'-dideoxypurine analogs

Virus	^a EC ₅₀ , μ M (fold-resistance)		
	AZT	3'-Azido-ddG	3'-Azido-ddA
WT	0.19 \pm 0.11	2.1 \pm 0.9	10.7 \pm 4.9
K65R	0.21 \pm 0.15 (1.1)	5.0 \pm 1.3 (2.3)	9.8 \pm 8.4 (0.9)
L74V	0.21 \pm 0.08 (1.1)	2.9 \pm 0.9 (1.4)	13.7 \pm 5.7 (1.2)
M184V	0.18 \pm 0.16 (1.0)	1.7 \pm 0.1 (0.8)	8.9 \pm 2.3 (0.8)
^b Q151M	213.7 \pm 12.3 (1,124)	72.9 \pm 29.5 (34.7)	70.1 \pm 10.8 (6.5)
^c TAM1	10.4 \pm 8.9 (54)	5.2 \pm 2.3 (2.5)	24.2 \pm 3.7 (2.2)
^d TAM2	11.9 \pm 11.6 (62)	3.7 \pm 1.4 (1.8)	19.4 \pm 8.1 (1.8)
^e TAM3	96.7 \pm 29.3 (507)	7.6 \pm 2.2 (3.5)	31.6 \pm 3.7 (2.9)
^f TAM4	58.6 \pm 9.2 (307)	7.9 \pm 4.9 (3.7)	37.5 \pm 6.2 (3.5)
^g 69SS	204.6 \pm 18.6 (1,076)	26.4 \pm 8.8 (12.5)	29.9 \pm 5.9 (2.8)

^a data are the mean \pm standard deviation of three independent experiments determined in P4/R5 cells [337].

^b Q151M Complex contains Q151M and also mutations A62V, V75I, F77L and F116Y.

^c M41L/L210W/T215Y.

^d D67N/K70R/T215F/K219Q.

^e M41L/D67N/K70R/T215F/K219Q.

^f M41L/D67N/K70R/L210W/T215Y/K219Q.

^g 69SS Insertion Complex consists of a dipeptide insertion (Ser-Ser) between codons 69 and 70 of RT together with mutations M41L/A62V/T69S/T215Y.

The 3'-azido-2',3'-dideoxypurine sub-class of NRTI are potent inhibitors of HIV-1 even in the presence of most RT-resistance mutations. Importantly, it should be noted that these compounds were previously identified, but overshadowed by the contemporary development of other compounds due initial toxicity studies [345-347]. The selected mutations and associated mechanisms resistance are not known for these compounds. We aim to delineate these properties *in vitro* to provide further evidence for the usefulness of this sub-class of NRTI.

2.0 HYPOTHESIS AND SPECIFIC AIMS

Drug resistance limits the usefulness of current NRTIs [348]. Resistance barriers and the degree of cross-resistance within the class of NRTI are variable. There is a clinical need to expand the repertoire of potent antiretroviral compounds that are active against drug-resistant HIV-1 RT. It has been shown that the base structure of a 3'-azido-2',3'-dideoxynucleoside influences its antiretroviral activity [337]. Of particular interest, AZT-resistant HIV-1 remains susceptible to 3'-azido-2',3'-dideoxypurine analogs (ADP). This finding may be exploited for rational drug design of novel NRTI with similar activities. The goals of this study are to characterize base-modified ADPs that are active against drug-resistant virus and to define the structural components of ADPs that are important for antiviral activity and resistance.

Cell-based and biochemical methods will be used to investigate a panel of 3'-azido-2',3'-dideoxynucleoside compounds with different base structures to determine the relationship between structure, activity and resistance. This will define chemical moieties that will aid in rationale drug design and development.

2.1 HYPOTHESES

Nucleoside analogs consisting of different base structures and identical 3'-azido-2',3'-dideoxyribose sugar structures will have different antiretroviral activities and will select for

different resistance mutations *in vitro*. The biochemical mechanisms of resistance by different mutations will differ based upon which mutations are selected. The cytidine and thymidine analogs, including the well-studied AZT, will have similar profiles and the guanosine and adenosine analogs will have similar profiles, but the profiles of the pyrimidine-based analogs will be markedly different than the purine-based analogs in respect to antiretroviral activities, selected resistance mutations and biochemical mechanisms of resistance.

2.2 SPECIFIC AIMS

2.2.1 Aim 1

To determine the antiretroviral activity and selected resistance 3'-azido-ddG, 3'-azido-ddC, or 3'-azido-ddA, Figure 17, will be tested in cell culture by single-cycle or multiple-cycle viral assays to determine median 50% inhibitory concentrations (EC₅₀), resistant virus will be produced by passaging virions through increasing concentrations of each individual nucleoside analog and the mutation profiles of the selected viruses will be examined. Population and single genome DNA sequencing will be used to identify mutations and the influence of each mutation on drug susceptibility and cross-resistance will be resolved using recombinant viruses generated by site-directed mutagenesis or cloning entire RT sequenced isolated from individual genomes. Structure-resistance relationships of base-modified ADP compounds will be defined with respect to their influence on both antiretroviral activity and selected resistance.

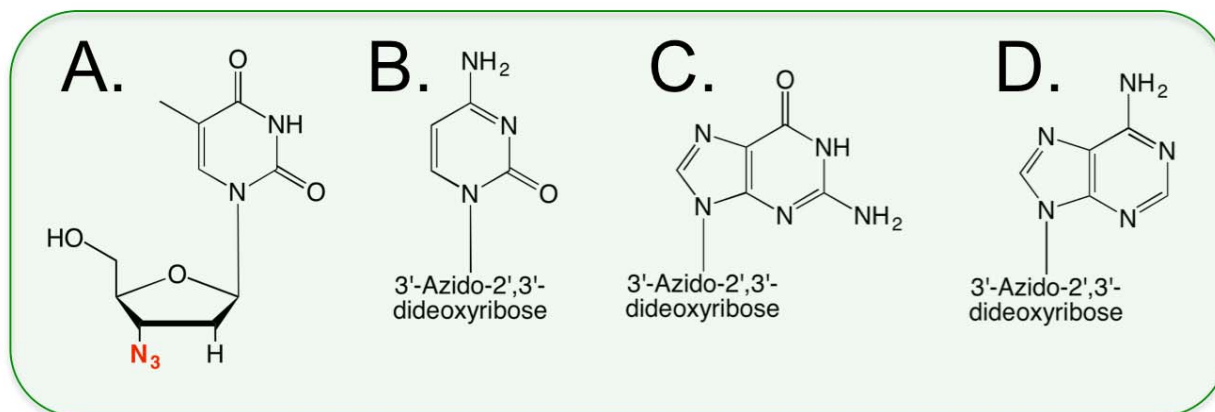


Figure 17: Structures of 3'-azido-2',3'-dideoxynucleoside analogs

A. 3'-azido-ddT (AZT), B. 3'-azido-ddC, C. 3'-azido-ddG, D. 3'-azido-ddA

2.2.2 Aim 2

Purified recombinant RT from site-directed mutagenesis and isolated RT sequence clones will be used to investigate the biochemical mechanisms of resistance. The purified enzymes will be assayed for steady-state polymerase activities, steady-state ATP-mediated excision, steady-state RNase H cleavage, pre-steady-state single-nucleotide incorporation and RT-Template/Primer binding affinity.

3.0 THE BASE COMPONENT OF 3'-AZIDO-2',3'-DIDEOXYNUCLEOSIDES INFLUENCES RESISTANCE MUTATIONS SELECTED IN HIV-1 REVERSE TRANSCRIPTASE

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3.1 PREFACE

This chapter is adapted with permission from a published study (Meter, J.D., Koontz, D., Asif, G. Zhang, H., Detorio, M., Solomon, S., Coats, S.J., Sluis-Cremer, N., Schinazi, R.F. and Mellors, J.W. 2011. The Base Component of 3'-Azido-2',3'-Dideoxynucleosides Influences Resistance Mutations Selected in HIV-1 Reverse Transcriptase. *Antimicrob Agents Chemother*, 55(8): p. 3758–3764). Copyright © 2011, American Society for Microbiology.

Additionally, this study was presented in part as an oral presentation and poster at the 17th International HIV Drug Resistance Workshop, Sitges, Spain, 2008 (Abstract 3, published in Meter, J., Koontz, D., Rapp, K.L., Detorio, M., Ruckstuhl, M., Schinazi, R.F., Mellors, J.W. Novel resistance profile of the potent nucleoside analogue reverse transcriptase inhibitor 3'-azido-2',3'-dideoxyguanosine. *Antiviral Therapy*, 2008, **13 Suppl 3**:A5); in part as a poster at the International HIV & Hepatitis Virus Drug Resistance Workshop & Curative Strategies, Dubrovnik, Croatia, June 2010 (Abstract 67 published in Meter, J., Koontz, D., Schinazi, R.F., Sluis-Cremer, N., Mellors, J.W. 2010. The base component of 3'-azido-2',3'-dideoxynucleosides determines the mutation patterns selected in HIV-1 reverse transcriptase. *Antiviral Therapy*, 2010, **15 Suppl 2**:A81).

The work presented in this chapter is in partial fulfillment of dissertation Aim 1. Jeffrey Meter performed all experimental work with the exception of AZT selection experiments completed by Dianna Koontz and the quantitation of metabolism of 3'-azido-2',3'-ddA by Ghazia Asif, Hong-wang Zhang, Mervi Detorio and Sarah Solomon.

3.2 ABSTRACT

We recently reported that HIV-1 resistant to 3'-azido-3'-deoxythymidine (AZT) is not cross-resistant to 3'-azido-2',3'-dideoxypurines. This finding suggested that the nucleoside base is a major determinant of HIV-1 resistance to nucleoside analogs. To further explore this hypothesis, we conducted *in vitro* selection experiments by serial passage of HIV-1_{LAI} in MT-2 cells in increasing concentrations of 3'-azido-2',3'-dideoxyguanosine (3'-azido-ddG), 3'-azido-2',3'-dideoxycytidine (3'-azido-ddC), or 3'-azido-2',3'-dideoxyadenosine (3'-azido-ddA). 3'-Azido-ddG selected for virus that was 5.3-fold resistant to 3'-azido-ddG compared to wild-type HIV-1_{LAI} passaged in the absence of drug. Population sequencing of the entire reverse transcriptase (RT) gene identified L74V, F77L, and L214F mutations in the polymerase domain and K476N and V518I mutations in the RNase H domain. However, when introduced into HIV-1 by site-directed mutagenesis, these 5 mutations only conferred ~2.0-fold resistance. Single-genome sequencing analyses of the selected virus revealed a complex population of mutants that all contained L74V and L214F linked to other mutations, including ones not identified during population sequencing. Recombinant HIV-1 clones containing RT derived from single sequences exhibited 3.2- to 4.0-fold 3'-azido-ddG resistance. In contrast to 3'-azido-ddG, 3'-azido-ddC selected for the V75I mutation in HIV-1 RT that conferred 5.9-fold resistance, compared to the wild-type virus. Interestingly, we were unable to select HIV-1 that was resistant to 3'-azido-ddA, even at concentrations of 3'-azido-ddA that yielded high intracellular levels of 3'-azido-ddA-5'-triphosphate. Taken together, these findings show that the nucleoside base is a major determinant of HIV-1 resistance mechanisms that can be exploited in the design of novel nucleoside RT inhibitors.

3.3 GOAL OF STUDY

Drug resistance limits the usefulness of current approved NRTI. Resistance barriers and the degree of cross-resistance within the class of NRTI are variable. There is a clinical need to expand the repertoire of potent antiretroviral compounds that are active against drug resistant HIV-1 RT. It was recently shown that the base structure of the 3'-azido-2',3'-dideoxynucleoside influenced its activity [337]. Of particular interest, AZT-resistant HIV-1 remains susceptible to purine analogs, 3'-azido-2',3'-ddG and 3'-azido-2',3'-ddA. This finding may be exploited for rational drug design of novel NRTI with similar activities. The goal of this study was to characterize the RT mutations selected by *in vitro* passage of virus in the presence of 3'-azido-nucleoside analogs and to define the importance of the mutations by site-directed mutagenesis.

3.4 MATERIALS AND METHODS

3.4.1 Nucleosides

3'-azido-ddA and 3'-azido-ddG were obtained from Berry Associates, Inc. (Ann Arbor, MI). AZT and ddI were obtained from Sigma Chemical Corporation (St. Louis, MO). ABC was obtained from GlaxoSmithKline (Research Triangle Park, NC). TFV was obtained from the AIDS Research and Reference Reagent Program (Division of AIDS, NIAID, NIH). 3'-azido-ddC, 3TC, and d4T were kindly provided by Raymond Schinazi (Emory University). 3'-azido-ddA-5'-triphosphate was synthesized as previously described [349]. All NRTIs were prepared as 40 mM stock solutions in dimethyl sulfoxide or sterile water and stored at -20°C.

3.4.2 Cells and viruses

MT-2 cells (AIDS Research and Reference Reagent Program) were cultured in RPMI 1640 with 2 mM l-glutamine (Lonza, Walkersville, MD) supplemented with 10% fetal bovine serum (HyClone Laboratories, Inc., Logan, UT), 10 mM HEPES buffer (Gibco, Grand Island, NY), and 50 IU/ml of penicillin and 50 µg/ml of streptomycin (Gibco). The P4/R5 reporter cell line (provided by Nathaniel Landau, Salk Institute, La Jolla, CA), which expresses the β -galactosidase gene under the control of the HIV-1 long terminal repeat promoter that is transactivated by HIV-1 tat, was maintained in phenol red-free Dulbecco's modified Eagle medium (Gibco) supplemented with 10% fetal bovine serum, 50 IU/ml of penicillin, 50 µg/ml of streptomycin, and 0.5 µg/ml of puromycin (Clontech, Palo Alto, CA). Stock viruses were prepared in MT-2 cells as described previously [336]. Briefly, 5 µg of plasmid DNA was electroporated into 1.3×10^7 MT-2 cells. Cell-free supernatants were collected 5 to 7 days posttransfection at peak cytopathic effect (CPE) and stored at -80°C . The infectivities of the virus stocks were determined by 3-fold end point dilution in P4/R5 cells, and the 50% tissue culture infectivity dose was calculated using the Reed and Muench equation [350].

3.4.3 Selection of drug-resistant HIV-1

Resistant virus was selected by serial passage of wild-type (WT) xxHIV_{LAI} in MT-2 cells in increasing concentrations of 3'-azido-ddG, 3'-azido-ddC, or 3'-azido-ddA. To initiate each selection experiment, MT-2 cells (1×10^6) were pretreated for 2 h with twice the concentration of drug required to inhibit viral replication by 50% (EC_{50}) of the nucleoside analog before inoculation with virus. Viral replication was monitored by CPE. At three or four syncytia per

field at 100× magnification, the cell-free supernatant was harvested and 0.1 ml of supernatant was added to fresh MT-2 cells to initiate a new passage. Remaining supernatant was stored frozen at −80°C. The concentration of drug was doubled every 5 to 15 passages, as viral growth permitted. Mean EC₅₀ values (n = 3) were determined at every five passages to identify changes in drug susceptibility. Fold resistance was calculated by dividing the mean EC₅₀ of the passaged virus by the mean EC₅₀ of wild-type HIV-1_{LAI}. The population genotype of the virus was determined every 10 passages by standard automated sequencing.

3.4.4 Drug susceptibility assays

NRTI susceptibility was determined in P4/R5 cells as described previously [336]. Briefly, 3-fold dilutions of inhibitor were added to P4/R5 cells in triplicate, and the cells were infected with an amount of virus that produced 100 relative light units (RLU) in no-drug virus control wells. After 48 h, the cells were lysed (Gal-Screen; Tropix/Applied Biosystems, Foster City, CA) and the RLU were measured using a ThermoLab Systems luminometer (Waltham, MA). The EC₅₀ and fold resistance were calculated as described above. 3'-azido-ddC is not well phosphorylated in P4/R5 cells, and the 3'-azido-ddC-selected virus did not grow well in P4/R5 cells. Susceptibility to 3'-azido-ddC was therefore determined in MT-2 cells, and viral replication was quantified by measuring p24 antigen production (Perkin-Elmer, Inc., Waltham, MA).

3.4.5 HIV-1 population sequencing

To confirm the genotypes of virus populations, viral RNA was extracted from culture supernatants by using oligo(dT)₂₅ Dynabeads (Invitrogen, Carlsbad, CA) according to the

manufacturer's instructions and treated with 1 IU/ μ l of DNase I for 2 h. The RNA was converted into cDNA and amplified using the SuperScript III one-step RT-PCR system with Platinum Taq DNA polymerase (Invitrogen). The entire coding region of RT was amplified using the forward primer 5'-GCTCTATTAGATACAGGAGCAGATGAT-3' and the reverse primer 5'-CCTTCTAAATGTGTACAATCTAGTTGCCAT-3'. PCR products were purified (Wizard SV Gel and PCR Clean-Up system; Promega, Madison, WI) and sequenced using a Big Dye terminator kit (v.3.1) on an ABI 3130 automated DNA sequencer (Applied Biosystems, Foster City, CA). The following forward primers were used for sequencing RT:

1. 5'-GGACCTACACCTGTCAAC-3'
2. 5'-GTTCCCTTAGATGAAGAC-3'
3. 5'-GAGGAACCAAAGCACTAA-3'
4. 5'-CACCCCTAACTGACACACC-3'

3.4.6 Construction of mutant recombinant HIV-1

Mutant RT clones were generated by site-directed mutagenesis (QuikChange Lightning site-directed mutagenesis kit; Stratagene, La Jolla, CA) using the p6HRT-MO plasmid. This plasmid contains the entire RT and protease coding sequence and four silent restriction sites (XmaI, MluI, XbaI, and NgoMIV from the 5' to 3' end of RT at codons 14, 358, 490, and 554, respectively) [286]. After site-directed mutagenesis, the mutated RT was ligated into pxxHIV-1_{LAI}-MO, which contains the entire genome of HIV-1_{LAI} and the same silent restriction sites in RT as p6HRT-MO. Infectious virus was generated by electroporation of the mutated xxHIV-1_{LAI}-MO plasmid into MT-2 cells as described above. All mutations in recombinant viruses were confirmed by

full-length sequencing of the entire RT coding region. Plasmids were kindly provided by Jessica Brehm (University of Pittsburgh).

3.4.7 SGS and generation of recombinant infectious viruses

Full-length RT sequences from single viral genomes in passaged virus were obtained by isolating viral RNA (as described above) and synthesizing cDNA with SuperScript III reverse transcriptase (Invitrogen) and primer 4232 (5'-TTCCCTACAATCCCCAAAGTCAAGG-3'). The cDNA was then serially diluted to yield 30% positive PCRs as described elsewhere for single-genome sequencing (SGS) [351]. First-round PCR was performed using AmpliTaq Gold (Applied Biosystems), primer 4232, and primer Bcl (5'-AGGAAGAATGGAAACCAAAAATGATAG-3'). Second-round PCR was performed using primers Bcl and 3908 (5'-CAAAAGAAATAGTAGCCAGCTGTG-3'). Positive reactions were determined by gel electrophoresis, purified by treatment with ExoSAP-IT (USB, Cleveland, OH), and sequenced as described above [351]. To generate infectious recombinant clones derived from single viral genomes, full-length RT was amplified from purified first-round PCR product using the following primers containing restriction sites:

1989Bcl, 5'-GTTTTATCAAAGTAAGACAGTATGATCAGATAC-3';

Sgr, 5'-TAACCTGCCACCGGTGGTAG-3'.

The purified products were concatemerized, digested with BclI and SgrAI, and cloned into pxxLAI-3D [pxxLAI-MO with 4 additional silent restriction sites: BclI (nucleotide [nt] 2011), BstBI (nt 3096), HpaI (nt 3383), and SgrAI (nt 3897)]. The recombinant plasmid was used to produce infectious virus as described above. Primers and plasmid were kindly provided by Jessica Brehm (University of Pittsburgh).

3.4.8 Cellular metabolism of 3'-azido-ddA

MT-2 cells (5×10^6) were incubated for 4 h with 3'-azido-ddA at 5, 12.5, 25, and 50 μ M 3'-azido-ddA. The cells were then centrifuged for 10 min at $350 \times g$ at 4°C, and the pellet was resuspended and washed three times with cold phosphate-buffered saline. Nucleoside triphosphates were extracted by incubation overnight at -20°C with 1 ml 60% methanol–water. The supernatants were then collected and centrifuged at $16,000 \times g$ for 5 min. The pellets were then reextracted for 1 h on ice by using an additional 200 μ l of 60% methanol in water, followed by centrifugation at $16,000 \times g$ for 5 min. The extracts were combined, dried under a gentle filtered airflow, and stored at -20°C. The residues were resuspended in 100 μ l of water prior to liquid chromatography (LC)-tandem mass spectrometry analysis.

The LC system was an UltiMate 3000 modular system (Dionex, Sunnyvale, CA) consisting of a quaternary pump, vacuum degasser, thermostated autosampler (4°C), and thermostated column compartment (28°C). An API5000 mass spectrometer (AB/SCIEX, Foster City, CA) was used as a detector. LC Analyst software version 1.4.2 was used to control both the LC and the mass spectrometer and for the data analysis and quantification. Phosphorylated 3'-azido-ddA was quantified by ion exchange, with the separation performed on a 5- μ m-particle-size Biobasic C18 (50- by 1.0-mm) column (Thermo Electron, Bellefonte, PA) using a gradient. The mobile phase consisted of A (10 mM ammonium acetate), B (ammonia buffer [pH 9.6]), and C, acetonitrile. Sample volumes of 5 μ l were injected onto the column.

The flow was diverted to waste for the initial 1 min of the analysis. Initial composition of the mobile phase was 70% A and 30% C with a gradient at 3.5 min to 70% B and 30% C for 5 min. The mass spectrometer was operated in positive ionization mode with a spray voltage of 5 kV, gas 1 at 15 (arbitrary units), gas 2 at 20 (arbitrary units), and source temperature of 230°C. A

standard curve was prepared by spiking 8 standards of synthesized 3'-azido-ddA-5'-TP in the range of 1 nM to 500 nM in cell lysate. The precursor-product ion transitions, declustering potential (DP, in V), collision energies (CE, in V), and exit potential (CXP, in V) for 3'-azido-ddA-DP and -TP were m/z 437.2 \rightarrow m/z 136; DP 121, CE 29, CXP 14; m/z 517.2 \rightarrow m/z 136; DP 121, CE 31, CXP 14, respectively.

3.5 RESULTS

3.5.1 Selection of HIV-1 resistance to 3'-azido-ddG

Wild-type HIV-1_{LAI} was serially passaged in MT-2 cells in increasing concentrations of 3'-azido-ddG. After every fifth passage, 3'-azido-ddG susceptibility of the passaged virus was determined in a single-cycle replication inhibition assay in P4/R5 cells, Figure 18. Full-length RT genotype analysis of the virus population (i.e., population genotype) was determined every 10th passage, Figure 18. L74V was the first mutation to emerge at passage 10, followed by L214F at passage 30, V518I at passage 60, and K476N and F77L at passage 90, Figure 18. The final virus population contained all 5 mutations and was 5.3-fold more resistant than a control xxHIV-1_{LAI} that was passaged in parallel in the absence of drug. No RT mutations were detected in the control virus.

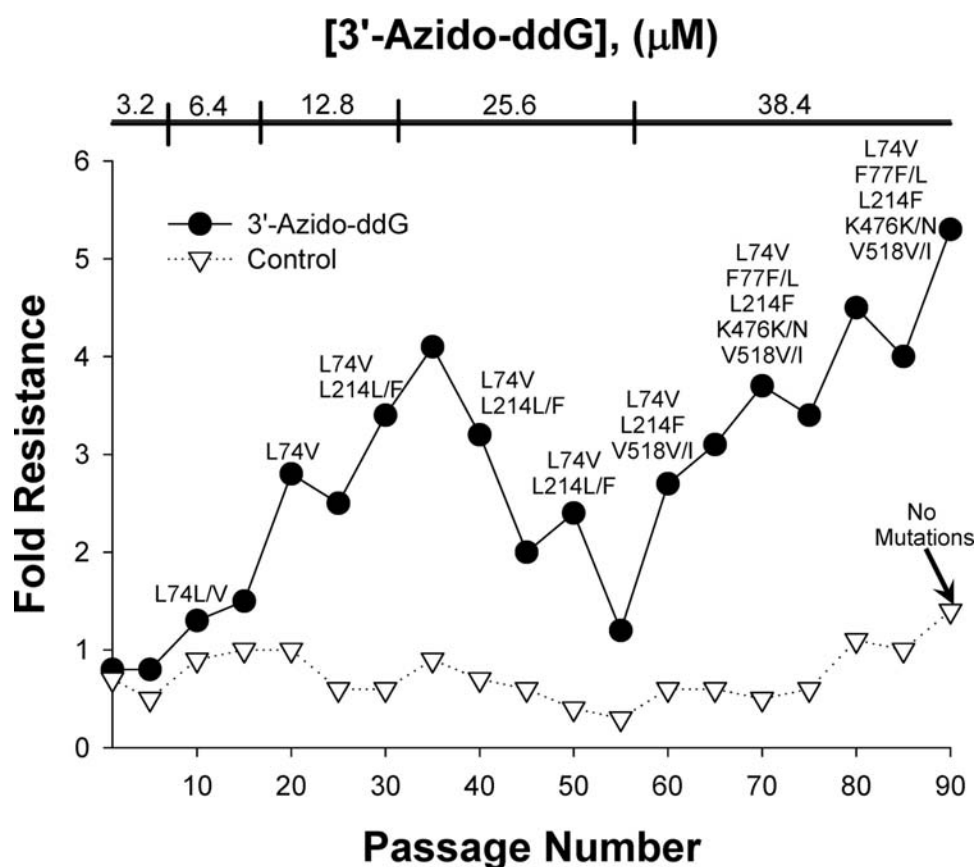


Figure 18: *In vitro* selection of 3'-azido-ddG resistant HIV-1

3'-azido-ddG-resistant xxHIV-1_{LAI} was selected by serial passage of virus in increasing concentrations of 3'-azido-ddG. Passaged virus was phenotyped for 3'-azido-ddG susceptibility (EC_{50} , $10.1 \pm 2.5 \mu\text{M}$ at passage 88) and compared to the control wild-type xxHIV-1_{LAI} passaged in parallel without 3'-azido-ddG (EC_{50} , $1.91 \pm 1.16 \mu\text{M}$ at passage 88) to determine resistance at every fifth passage. Standard population sequencing was performed at every 10th passage and identified the indicated RT mutations that differed from the starting virus population.

Next, we tested the susceptibility of 3'-azido-ddG-resistant virus from passage 88 to a panel of NRTIs in P4/R5 cells, Table 5. The 3'-azido-ddG-resistant HIV-1 showed cross-resistance to ddI (6.2-fold), 3TC (4.8-fold), and ABC (4.2-fold). Low-level cross-resistance was also noted for TFV (2.5-fold), D4T (1.5-fold), and 3'-azido-ddA (1.5-fold). 3'-azido-ddG-resistant HIV-1 remained sensitive to AZT.

Table 5: Resistance and cross-resistance of wild-type (WT) and 3'-azido-ddG-selected virus

Drug	^a EC ₅₀ , μ M		^d Fold Resistance	^e <i>P</i> value
	^b WT	^c Selected Virus		
ddI	4.3 \pm 0.2	26.6 \pm 1.2	6.2	<0.0001
3'-azido-ddG	1.9 \pm 1.2	10.1 \pm 2.5	5.3	<0.001
3TC	2.2 \pm 1.8	10.5 \pm 4.1	4.8	<0.05
ABC	8.9 \pm 0.5	37.2 \pm 6.3	4.2	<0.05
TFV	2.1 \pm 0.7	5.4 \pm 0.7	2.5	<0.05
d4T	14.1 \pm 3.0	23.2 \pm 1.1	1.5	<0.01
3'-azido-ddA	3.7 \pm 0.6	5.6 \pm 0.2	1.5	<0.05
AZT	0.3 \pm 0.2	0.4 \pm 0.3	1.3	NS

^a Mean \pm standard deviation of three experiments.

^b WT virus passaged in parallel with the 3'-azido-ddG selection virus in the absence of drug; genotype confirmed by sequencing.

^c Virus isolated from passage 88 of the 3'-azido-ddG selection that contained L74V, F77L, L214F, K476N, and V518I by standard population sequencing.

^d Fold resistance determined compared with wild-type xxHIV-1_{LAI}.

^e Statistical significance compared to wild-type xxHIV-1_{LAI} determined by two-sample Student's *t* test. NS, not significant.

3.5.2 3'-azido-ddG susceptibilities of viruses containing different combinations of L74V, F77L, L214F, K476N, and V518I

Recombinant viruses were generated by site-directed mutagenesis to contain different combinations of the L74V, F77L, L214F, K476N, and V518I mutations. Surprisingly, many of the recombinant viruses (e.g., K476N, L74V/L214F/K476N/V518I, and L74V/F77L/L214F/K476N/V518I) were growth defective, precluding further assessments of NRTI susceptibility, Table 6. Drug susceptibility assays in P4/R5 cells revealed that the F77L, L214F, and V518I mutations alone did not confer 3'-azido-ddG resistance, Table 6. Interestingly, the remaining recombinant viruses only displayed low levels (1.5- to 2.1-fold over WT) of 3'-azido-ddG resistance. This finding suggested that population sequencing did not provide an

adequate representation of the mutant variants within the selected virus population. Specifically, the results suggested that mutations identified by population sequencing showed variable linkage on viral genomes and that other important mutations may not have been detected.

Table 6: 3'-Azido-ddG and 3'-azido-ddC susceptibilities of HIV-1_{LAI} mutants

^a Virus	^b EC ₅₀ , μ M	^c Fold-Resistance	^d P value
<i>3'-Azido-ddG susceptibility</i>			
Wild-type	1.5 \pm 0.4	-	-
L74V	2.2 \pm 0.5	1.5	NS
L74V/L214F	2.8 \pm 0.4	1.9	<0.05
L74V/L214F/K476N	3.1 \pm 0.3	2.1	<0.005
L74V/L214F/V518I	2.2 \pm 0.9	1.5	NS
L74V/F77L/L214F/K476N	2.8 \pm 0.2	1.9	<0.05
^e K476N	-	-	-
^e L74V/L214F/K476N/V518I	-	-	-
^e L74V/F77L/L214F/K476N/V518I	-	-	-
<i>3'-Azido-ddC susceptibility</i>			
Wild-type	0.6 \pm 0.2	-	-
V75I	1.6 \pm 0.8	2.6	<0.05

^a Mutants were created in pxxLAI-MO by site-specific mutagenesis and infectious virus was produced by transfection of MT-2.

^b Mean \pm standard deviation of three experiments.

^c Fold resistance determined against wild-type xxHIV-1_{LAI-MO} clone.

^d Statistical significance compared to wild-type xxHIV-1_{LAI-MO} determined by two-sample Student's *t* test. NS, not significant.

^e The mutant virus was growth defective.

3.5.3 Single-genome sequencing and cloning of RT from the 3'-azido-ddG-resistant HIV-1

We performed single-genome sequencing analyses of full-length RT from virus at passage 90 to genetically characterize individual mutant variants within the selected virus population. This approach permitted more detailed analysis of selected viruses, including mutation linkage. Table 7 shows the mutational patterns observed in 22 single genome sequences. Interestingly, all of the sequences contained the L74V and L214F mutations. The F77L (91% of sequences), K476N

(68%), and V106I (50%) mutations were also frequently observed in the single genome sequences. By contrast, the frequencies of other mutations varied considerably (Table 7), and no obvious pattern of linkage emerged from this study.

Table 7: Predicted amino acid changes from wild-type RT in single genome sequences derived from 3'-azido-ddG-selected virus (passage 90)

RT residue	Sequence no.																						% of clones
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	
L74	V	V	V	V	V	V	V	V	V	V	V	V	V	V	V	V	V	V	V	V	V	V	100
F77	L	L	L	L		L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L		91
V106	I	I	I	I		I			I		I	I				I		I			I		50
E122	K	K				K	K				K					K		K			K		36
E169									K														5
L214	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	100
R277	K		K										K	K					K				23
R284											K												5
G333					E																		5
V435													I										5
S447	N					N													N				14
R461								K															5
K476		N	N	N	N		N		N	N		N		N		N	N	N		N	N	N	68
P510						T							T						T				14
K512																						R	5
E514												D											5
V518	I			I					I		I		I								I		27
V531	I			G																			5 I/G
L533				S		M																	5 S/M

Recombinant infectious viruses were created by ligating single-genome-derived full-length RT amplicons into a wild-type vector (pxxLAI-3D) to assess the phenotype of the different RT sequences. Infectious virus was produced and assayed for 3'-azido-ddG susceptibility in P4/R5 cells, Table 8. Some clones (36%) were growth defective and could not be phenotyped. All of the replication-competent viruses showed similar levels of 3'-azido-ddG resistance, with an average fold resistance of 3.6 ± 0.3 (range, 3.2 to 4.0). Of note, bulk cloning

of full-length RT amplified from the virus population into pxxLAI-3D produced virus exhibiting only 2.2-fold resistance to 3'-azido-ddG.

Table 8: 3'-Azido-ddG susceptibility of recombinant HIV-1 with RT derived from single-genome amplifications

^a Clone	^b Genotype	^c EC ₅₀ , μ M	^d Fold Resistance	^e P value
1	L74V/F77L/V106I/E122K/L214F/R277K/S447N/V518I/V531I	4.5 \pm 1.2	3.2	<0.05
2	L74V/F77L/V106I/E122K/L214F/K476N	4.8 \pm 1.0	3.4	<0.01
3	L74V/F77L/V106I/L214F/R277K/K476N	5.3 \pm 1.8	3.8	<0.05
6	L74V/F77L/V106I/E122K/L214F/S447N/P510T/L533M	5.0 \pm 1.6	3.6	<0.05
7	L74V/F77L/E122K/L214F/K476N	4.7 \pm 0.7	3.4	<0.01
^f 10	L74V/F77L/L214F/K476N	5.6 \pm 1.5	4.0	<0.01
13	L74V/F77L/L214F/R277K/V435I/P510T/V518I	5.4 \pm 0.9	3.8	<0.01
"Bulk"	Mixture	2.31 \pm 0.01	2.2	<0.05

^a Recombinant clones were produced by amplifying full length RT from single genomes or as a population (bulk) and cloning into the xxHIV_{LAI-3D} vector for infectious virus production by electroporation into MT-2 cells.

^b Genotypes identified by single genome sequencing (Table 7) that are not listed were cloned, but determined to be growth defective.

^c Mean \pm standard deviation of three experiments.

^d Fold resistance determined against xxHIV-1_{LAI} clone (EC₅₀ = 1.40 \pm 0.05 μ M)

^e Statistical significance compared to wild-type xxHIV-1_{LAI-MO} determined by two-sample Student's *t* test.

^f Clone 10 contains a silent G to A transition at nucleotide 423 that encodes for G141 in the HIV-1_{xxLAI} background.

3.5.4 Selection of HIV-1 resistance to 3'-azido-ddC

Following 55 passages of HIV-1_{LAI} in increasing concentrations of 3'-azido-ddC, we selected virus that was 5.9-fold resistant to 3'-azido-ddC compared to wild-type virus, Figure 19. Population sequencing of the entire HIV-1 RT identified the V75I mutation in the DNA polymerase domain of the enzyme. When introduced into the backbone of the wild-type HIV-

1_{LAI} virus by site-directed mutagenesis, the V75I mutation conferred 2.6-fold resistance to 3'-azido-ddC in MT-2 cells, Table 6.

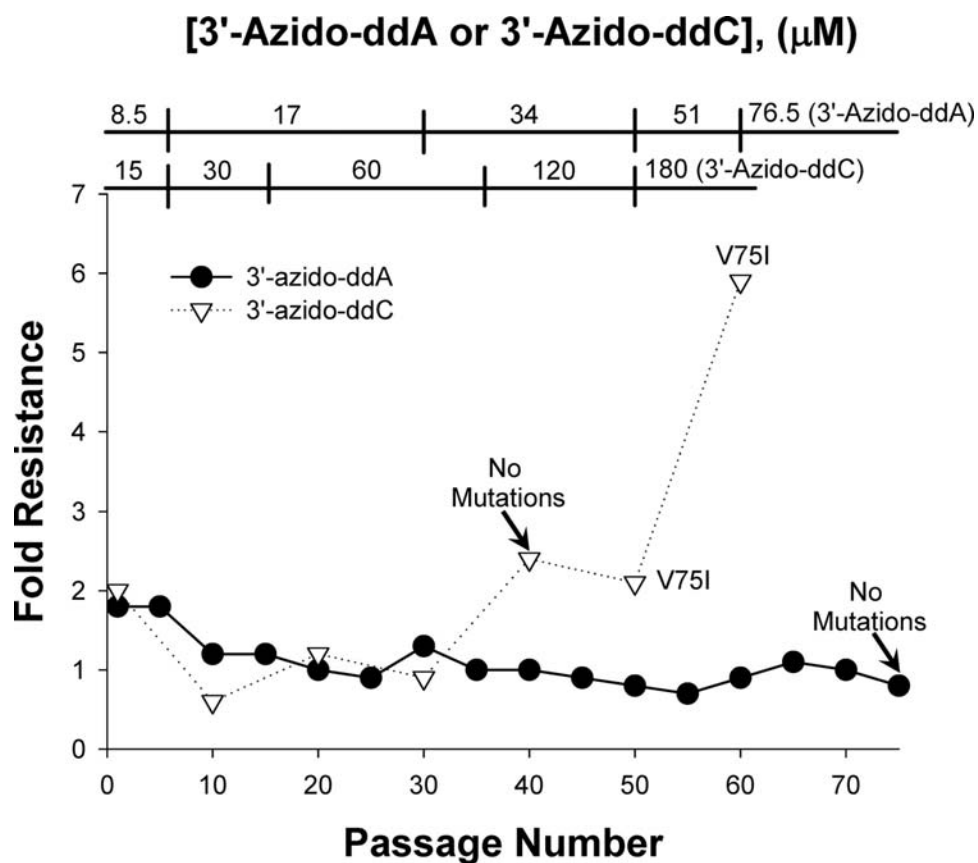


Figure 19: *In vitro* selection of 3'-azido-ddC and 3'-azido-ddA resistant HIV-1

Drug-resistant xxHIV-1_{LAI} was selected by serial passage of virus in increasing concentrations of 3'-azido-ddC (EC₅₀, 86.7 ± 16.9 μM at passage 60) or 3'-azido-ddA (EC₅₀, 3.32 ± 1.06 μM at passage 75). Fold resistance and RT genotype were determined as described for Figure 18.

3.5.5 Selection of HIV-1 resistance to 3'-azido-ddA

After 75 passages of HIV-1_{LAI} in increasing concentrations of 3'-azido-ddA (8.5 μM to 76.5 μM), we were unable to select for HIV-1 resistance to 3'-azido-ddA, Figure 19. This inability to

select for drug-resistant HIV-1 was not due to inefficient metabolism of 3'-azido-ddA to 3'-azido-ddATP. In Table 9, we show from the quantitative mass spectrometry analyses that 3'-azido-ddA was metabolized to the active TP form in MT-2 cells in a dose-dependent manner.

Table 9: Levels of 5'-phosphorylated 3'-azido-ddA in MT-2 cells after 4 h of incubation with 3'-azido-ddA

Extracellular 3'-azido-ddA (μM)	^a Level of metabolized 3'-azido-ddA (pmol/ 10^6 cells)		^d Level of metabolized 3'-azido-ddA (μM)	
	^b TP	^c DP	TP	DP
5	0.13 ± 0.02	0.030 ± 0.002	0.240 ± 0.004	0.054 ± 0.003
12.5	0.22 ± 0.01	0.08 ± 0.01	0.40 ± 0.02	0.15 ± 0.02
25	0.36 ± 0.05	0.17 ± 0.04	0.66 ± 0.09	0.30 ± 0.07
50	0.77 ± 0.07	0.25 ± 0.07	1.41 ± 0.14	0.45 ± 0.13

^a Mean \pm standard deviation of three experiments.

^b TP is 3'-azido-ddA-5'-triphosphate.

^c DP is 3'-azido-ddA-5'-diphosphate.

^d Mean \pm standard deviation of three experiments, determined using average diameter for MT-2 cells of 12.3 μm .

3.6 DISCUSSION

This study clearly shows that the base component of 3'-azido nucleosides strongly influences the pattern of resistance mutations in HIV-1 RT that are selected *in vitro*. Previously, we reported that AZT selected for the classical thymidine analog mutations D67N, K70R, and T215F in the DNA polymerase domain of the enzyme as well as A371V and Q509L in the connection and RNase H domains, respectively [286]. The experimental approach used previously to select AZT-resistant HIV-1 was essentially the same as that described here. In contrast to AZT, 3'-azido-ddG selected for different combinations of L74V, F77L, and L214F in the DNA polymerase and K476N and V518I in the RNase H domains of HIV-1 RT, respectively, and 3'-

azido-ddC selected only the V75I mutation in the DNA polymerase domain. Of note, we have been unable to select for 3'-azido-ddA resistance in MT-2 cells (Figure 19) or in primary human lymphocytes (data not shown).

The level of resistance selected *in vitro* to 3'-azido-ddG is lower than that observed for AZT. AZT-resistant HIV-1, selected after 65 passages in MT-2 cells, contained up to 5 mutations and exhibited >16,200-fold resistance to AZT [286]. By contrast, 3'-azido-ddG-resistant HIV-1, selected after 90 passages in MT-2 cells, also contained up to 5 mutations, but it exhibited only 5.3-fold resistance to 3'-azido-ddG. Interestingly, the virus population selected by 3'-azido-ddG was exceptionally diverse (Table 7), and the resistant phenotype could not be recapitulated through the construction of site-directed mutants that were based on the population genotype (Table 6). This finding suggests that population genotype analyses may not be definitive when complex mixtures of viruses are present because mutation linkage cannot be assessed. Indeed, our phenotypic analyses of single genome sequences provided a more accurate characterization of the resistant variants that emerged under 3'-azido-ddG selective pressure (Table 8). Importantly, these analyses also demonstrated that no single mutation or set of mutations is able to confer sufficient 3'-azido-ddG resistance such that it can become the dominant species in the population.

To date, the phenotypic mechanisms responsible for 3'-azido-ddG and 3'-azido-ddC resistance have not been elucidated, although ongoing biochemical studies are addressing this issue. The selection of the L74V and F77L mutations (part of the Q151M complex) by 3'-azido-ddG likely suggests a discrimination mechanism. However, the mechanisms of resistance for K476N and V518I are uncertain. Both residues reside in the RNase H domain and may interact with the T/P positioning or affect RNase H cleavage activity. Figure 20 shows the locations of

these mutations in the crystal structure of HIV-1 RT. Of note, other resistance mutations in the connection (e.g., N348I and A360V) and RNase H (e.g., Q509L) domains of HIV-1 RT influence drug susceptibility via an indirect RNase H-mediated effect on the NRTI-MP excision phenotype [340,352-355]. However, we have also shown that a mutation in the connection domain of HIV-1 RT (G333E) directly impacts the enzyme's ability to incorporate 3TC-TP [356].

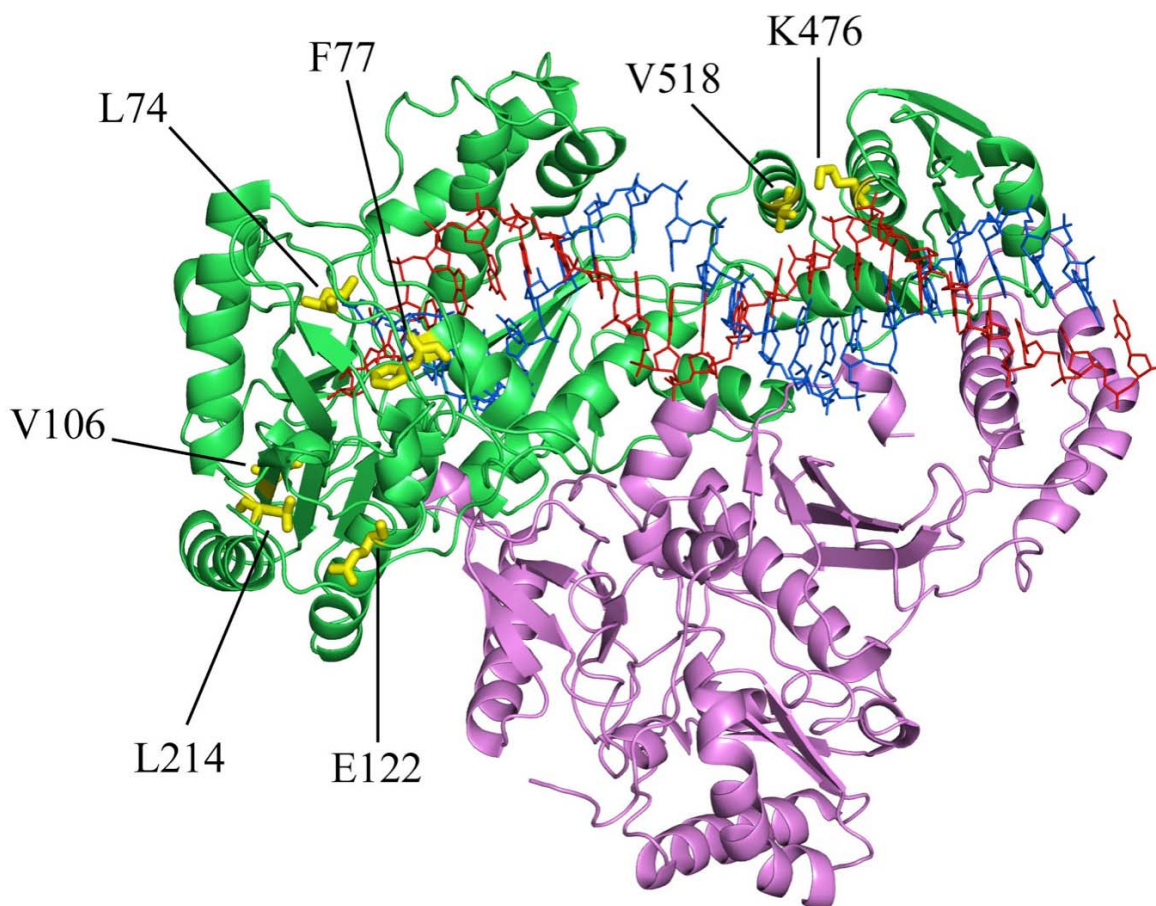


Figure 20: Locations of 3'-azido-ddG resistance mutations in RT (1HYS)

The p66 subunit and p51 subunit are shown as ribbon structures in green and pink, respectively. The residues are displayed as ball and stick models in yellow. The DNA primer strand and RNA template strand are represented in blue and red, respectively. Molecular graphics images were produced using POLYVIEW-3D Porollo A, Meller J: Versatile Annotation and Publication Quality Visualization of Protein Complexes Using POLYVIEW-3D, BMC Bioinformatics 2007, **8**: 316 and rendered using The PyMOL Molecular Graphics System, Version 1.5.0.4 Schrödinger, LLC.

The V75I mutation identified in the 3'-azido-ddC-resistant HIV-1 has also been observed in selection experiments with acyclovir (ACV) and a monophosphorylated prodrug of ACV [357,358]. Biochemical studies showed that mutant V75I RT did not alter binding efficiency of ACV-triphosphate but increased incorporation of dGTP versus ACV-5'-triphosphate through a discrimination mechanism [358]. This suggests that a discrimination mechanism may also be responsible for the observed HIV-1 resistance to 3'-azido-ddC. Studies of cross-resistance of the V75I mutant in primary activated CD4+ lymphoblasts have shown that V75I increases the EC50 for FTC, 3TC, ddI, and ABC, does not change the EC50 for TDF or d4T, and causes hypersusceptibility to AZT [359].

We were unable to select 3'-azido-ddA-resistant HIV-1 *in vitro*. Virus from passage 75 in media containing 3'-azido-ddA concentrations up to 75.6 μ M did not show mutations in RT and did not exhibit any change in 3'-azido-ddA susceptibility. It is not clear why resistance did not emerge and how passaged virus was able to replicate despite high 3'-azido-ddA concentrations. We showed that 3'-azido-ddA was efficiently metabolized to the active 5'-triphosphate form in MT-2 cells that were used for selection (Table 9). Nevertheless, there may be subsets of cells that do not efficiently metabolize 3'-azido-ddA to the active 5'-triphosphate and allow virus to replicate without developing resistance. Such a mechanism has been shown *in vitro* through isolation of cell clones that are not protected from HIV-1 infection by AZT [360]. The cell cycle can transiently alter the amount of intracellular NRTI-TP or the levels of competing natural dNTPs. Cellular resistance, or permanent alterations in NRTI metabolism or uptake, is more likely to develop during prolonged exposure of cells to an NRTI [361]. We reduced this latter possibility by using new cells at the start of each passage, although the 5- to 7-day duration of each passage could have allowed outgrowth of cells with altered NRTI metabolism. An

alternative explanation for HIV-1 replication in the presence of 3'-azido-ddA is the emergence mutations outside of RT that could increase viral infectivity such that a subset of cells with subinhibitory 3'-azido-ddATP concentrations could be infected efficiently.

In summary, NRTIs with different base structures, but the same 3'-azido-2',3'-dideoxyribose sugar, selected for divergent resistance mutations in RT *in vitro*. These findings indicate that the base component of NRTIs can be modified to alter the mechanisms and genetic barrier to HIV-1 drug resistance. These insights should prove useful in the synthesis of novel NRTIs that pose a high genetic barrier to HIV-1 resistance.

4.0 MOLECULAR MECHANISM OF HIV-1 RESISTANCE TO 3'-AZIDO-2',3'- DIDEOXYGUANOSINE

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4.1 PREFACE

This chapter is adapted from an unpublished study (Meteer, J.D., Sluis-Cremer, N., Schinazi, R.F. and Mellors, J.W. 2013. Molecular mechanism of HIV-1 resistance to 3'-azido-2',3'-dideoxyguanosine.). The work presented in this chapter is in partial fulfillment of dissertation Aim 2. Jeffrey Meteer performed all experimental work in this study.

4.2 ABSTRACT

We recently reported that 3'-azido-ddG selected for the L74V, F77L, and L214F mutations in the polymerase domain and K476N and V518I mutations in the RNase H domain of HIV-1 RT. In this study, we have defined the molecular mechanisms of 3'-azido-ddG resistance by performing in-depth biochemical analyses of HIV-1 RT containing mutations L74V/F77L/V106I/L214F/R277K/K476N (SGS3). The SGS3 HIV-1 RT was from a single-genome-derived full-length RT sequence obtained from 3'-azido-ddG resistant HIV-1 selected *in vitro*. We also analyzed two additional constructs that either lacked the L74V mutation (SGS3-L74V) or the K476N mutation (SGS3-K476N). Pre-steady-state kinetic experiments revealed that the L74V mutation allows HIV-1 RT to effectively discriminate between the natural nucleotide (dGTP) and 3'-azido-ddG-triphosphate (3'-azido-ddGTP). 3'-azido-ddGTP discrimination was primarily driven by a decrease in 3'-azido-ddGTP binding affinity (K_d) and not by a decreased rate of incorporation (k_{pol}). The L74V mutation was found to severely impair RT's ability to excise the chain-terminating 3'-azido-ddG-monophosphate (3'-azido-ddGMP) moiety. However, the K476N mutation partially restored the enzyme's ability to excise 3'-azido-

ddGMP on an RNA/DNA, but not on DNA/DNA, template/primer by selectively decreasing the frequency of secondary RNase H cleavage events. Taken together, these data provide strong additional evidence that the nucleoside base structure is major determinant of HIV-1 resistance to the 3'-azido-2',3'-dideoxynucleosides.

4.3 GOAL OF STUDY

AZT typically selects for TAMs in HIV-1 [199,200]. AZT-MP is also more readily excised by HIV-1 RT containing TAMs than are other sugar modified NRTI-MP analogs [362]. Initially, Boyer *et al.* proposed that this was due to the 3'-azido group, which anchored the chain-terminating AZT-MP in the excision-competent nucleotide-binding site (N-site) and prevented its translocation to the excision-incompetent primer-binding site (P-site) [363]. However, we reported that the 3'-azido-2',3'-dideoxypurines retained activity against HIV-1 variants that contained multiple TAMs [337,344]. This finding suggested that the 3'-azido-2',3'-dideoxynucleoside base was a major determinant of HIV-1 resistance. To further explore this hypothesis, we conducted *in vitro* selection experiments by serial passage of HIV-1_{LAI} in MT-2 cells in increasing concentrations of 3'-azido-ddG [364]. 3'-Azido-ddG selected for virus that was 5.3-fold resistant to the nucleoside compared to wild-type (WT) HIV-1_{LAI} passaged in the absence of drug. Population sequencing of the entire reverse transcriptase (RT) gene identified L74V, F77L and L214F mutations in the polymerase domain and K476N and V518I mutations in the RNase H domain. Under similar conditions, AZT selected for highly resistant virus (>16,200-fold over WT) that contained the TAMs D67N, K70R, T215F, A371V and Q509L in

RT [286,340]. The selection of divergent mutations indicates that the phenotypic mechanisms responsible for resistance between 3'-azido-ddG and AZT are different.

We therefore investigated the molecular mechanisms of resistance to 3'-azido-ddG by performing in-depth biochemical analyses of wild-type and mutants HIV-1 RTs containing L74V, L74V/F77L/V106I/L214F/R277K/K476N (SGS3), F77L/V106I/L214F/R277K/K476N (SGS3 Δ L74V) and L74V/F77L/V106I/L214F/R277K (SGS3 Δ K476N). We report that the L74V mutation allows HIV-1 RT to effectively discriminate between the natural nucleotide (dGTP) and 3'-azido-ddG-triphosphate (3'-azido-ddGTP). We also show that the K476N mutation partially restores the enzyme's ability to excise 3'-azido-ddGMP on an RNA/DNA, but not DNA/DNA, template/primer by selectively decreasing the frequency of secondary RNase H cleavage events.

4.4 MATERIALS AND METHODS

4.4.1 Materials

AZT-TP and 3'-azido-ddGTP were purchased from Trilink Biotechnologies (San Diego, CA). ATP, deoxyribonucleotide triphosphates (dNTPs) and dideoxy nucleoside triphosphates were purchased from GE Healthcare (Piscataway, New Jersey, USA), and [γ - 32 P]-ATP was acquired from PerkinElmer Life Sciences (Boston, Massachusetts, USA). RNA and DNA oligonucleotides were synthesized by Integrated DNA Technologies (Coralville, Iowa, USA).

4.4.2 Cloning, site-directed mutagenesis and purification of HIV-1 RT

We previously reported that when the L74V, F77L, L214F, K476N and V518I mutations were introduced into HIV-1 by site-directed mutagenesis they only conferred ~2.0-fold resistance [364]. However, if we generated HIV-1 clones containing single-genome-derived full-length RT sequences from the 3'-azido-ddG resistant virus population selected *in vitro*, the recombinant virus yielded higher levels of 3'-azido-ddG resistance (range 3.2 to 4.0-fold) [364]. Therefore, in this study we cloned into the p6HRT-PROT prokaryotic expression vector [365] one of these single-genome-derived full-length RT sequences (SGS3) that contained L74V/F77L/V106I/L214F/R277K/K476N mutations. The contributions of the L74V and K476N mutations were studied in the context of SGS3 HIV-1 RT by reverting out the mutations by site-directed mutagenesis (QuikChange Lightning site-directed mutagenesis kit; Stratagene, La Jolla, CA) to generate the F77L/V106I/L214F/R277K/ K476N (SGS3 Δ L74V) and L74V/F77L/V106I/L214F/R277K (SGS3 Δ K476N) enzymes. We also introduced the L74V mutation into WT HIV-1_{LAI} RT by site-directed mutagenesis. Full-length sequencing of mutant RTs was performed to confirm the presence of the desired mutations and to exclude adventitious mutations introduced during mutagenesis. WT and mutant recombinant HIV-1 RTs were over-expressed and purified to homogeneity as described previously [365]. RT concentration was determined spectrophotometrically at 280nm using an extinction co-efficient (ϵ_{280}) of 260 450 M⁻¹ cm⁻¹.

4.4.3 Steady-state DNA polymerization by WT & mutant HIV-1 RT

A 19 nucleotide DNA primer (P19; 5'-TTGTAGCACCATCCAAAGG-3') annealed to a 36 nucleotide DNA template (T36; 5'-AGAGCCCCCGAGACCTTTGGATGGTGCTACAAGCT-3') was used in these experiments. P19 was 5'-radiolabeled with [γ - 32 P]-ATP and T4 polynucleotide kinase, as described previously [233,337,340,366,367]. 5'- 32 P-labeled P19 was then annealed to T36 by adding a 1:1.5 molar ratio of primer to template at 90°C and allowing the mixture to slowly cool to ambient room temperature. DNA polymerization was assessed by incubating 200 nM WT or mutant HIV-1 RT with 20 nM template/primer (T/P; T36/P19) in 50mM Tris-HCl (pH 7.5), 50 mM KCl, 10 mM MgCl₂. The reaction was initiated by the addition of 0.1 or 1 μ M mixed dNTPs. After defined incubation periods, aliquots were removed and processed as described previously [337,340,366,367].

4.4.4 Steady-state assays of 3'-azido-ddGTP incorporation and 3'-azido-ddGMP excision by WT and mutant HIV-1 RT

In these assays, we assessed the ability of WT or mutant HIV-1 RT to synthesize full-length DNA product on the T36/P19 T/P in the presence of 5 μ M 3'-azido-ddGTP and 3 mM ATP. Briefly, 200 nM WT or mutant HIV-1 RT was pre-incubated with 20 nM 5'- 32 P-end-labeled T/P in 50mM Tris-HCl (pH 7.5), 50 mM KCl, 10 mM MgCl₂. Reactions were initiated by the addition of 0.5 μ M mixed dNTPs, 5 μ M 3'-azido-ddGTP and 3 mM ATP. After defined incubation periods, aliquots were removed and processed as described above.

4.4.5 Pre-steady-state assays of dGTP or 3'-azido-ddGTP incorporation by WT or mutant HIV-1 RT

A 5'-³²P-labeled 20 nucleotide DNA primer (P20; 5'-TCGGGCGCCACTGCTAGAGA-3') annealed to a 52 nucleotide DNA template (T36; 5'-CTCAGACCCTTTTAGTCAGAATG GAAAATCTCTAGCAGTGGCGCCCGAACAG-3') was used in these experiments. A Kintek RQF-3 instrument (Kintek Corporation, Clarence, PA) was used for pre-steady state experiments with reaction times ranging from 5 ms to 3 min. The typical experiment was performed at 37°C in 50 mM Tris-HCl (pH 7.5) containing 50 mM KCl, 10 mM MgCl₂ and varying concentrations of dGTP or 3'-azido-ddGTP (0.5 to 10 μM). All concentrations reported refer to the final concentrations after mixing. WT or mutant HIV-1 RT (200 nM) was pre-incubated with 20 nM T/P, prior to rapid mixing with nucleotide and divalent metal ions to initiate the reaction that was quenched with 50 mM EDTA. Products were resolved and analyzed, as described previously [337,367]. Data were fitted by nonlinear regression with Sigma Plot software (Systat Software, Inc., San Jose, CA) using the appropriate equations [368]. The apparent burst rate constant (k_{obs}) for each particular concentration of dGTP or 3'-azido-ddGTP was determined by fitting the time courses for the formation of product using the following equation: $product = A[1 - \exp(-k_{obs}t)]$, where A represents the burst amplitude. The turnover number (k_{pol}) and apparent dissociation constant for the nucleotide analog (K_d) were then obtained by plotting the apparent catalytic rates (k_{obs}) against nucleotide analog concentrations and fitting the data with the following hyperbolic equation: $k_{obs} = (k_{pol}[dNTP])/([dNTP] + K_d)$. Catalytic efficiency was calculated as the ratio of turnover number over dissociation constant (k_{pol}/K_d). Selectivity for natural dGTP versus 3'-azido-ddGTP was calculated as the ratio of catalytic efficiency of dGTP over that of the analog $(k_{pol}/K_d)^{dGTP}/(k_{pol}/K_d)^{3'-azido-ddGTP}$.

4.4.6 Steady-state excision of 3'-azido-ddGMP by WT or mutant HIV-1 RT

A 23 nucleotide primer (P23; 5'-TTGTAGCACCATCCAAAGGTCTC-3') was 5'-end labeled with [γ - 32 P]-ATP, chain-terminated with 3'-azido-ddGMP and annealed to a DNA (T36) or RNA (T36^{RNA}; 5'-rCrArGrArGrCrCrCrCrCrGrArGrArCrCrUrUrUrGrGrArUrGrGrUrGrCrUrArCrArArGrCrU-3') template, as described previously [337,344,367]. 200 nM WT or mutant HIV-1 RT was pre-incubated with 20 nM T/P in 50mM Tris-HCl (pH 7.5), 50 mM KCl, 10 mM MgCl₂. Reactions were initiated by the addition of 2 μ M dGTP, 40 μ M ddCTP and 3 mM ATP. After defined incubation periods, aliquots were removed and processed as described above.

4.4.7 Assay for RT RNase H activity

WT and mutant RT RNase H activity was evaluated using the same 3'-azido-ddGMP chain-terminated RNA/DNA T/P substrate described above, except the 5'-end of the RNA was 32 P-end-labelled. Assays were carried out using 20 nM T_{RNA}/P_{3'-azido-ddG}, 3 mM ATP and 10 mM MgCl₂ in a buffer containing 50 mM Tris-HCl (pH 7.5) and 50 mM KCl. Reactions were initiated by the addition of 200 nM WT or mutant HIV-1 RT. Aliquots were removed, quenched at varying times, and analysed as described above.

4.5 RESULTS

4.5.1 DNA polymerase activity of WT and mutant HIV-1 RT

As described in the Materials and Methods, we cloned a single-genome-derived full-length RT sequence (SGS3) derived from a 3'-azido-ddG resistant virus population selected *in vitro* [364] that contained the L74V/F77L/V106I/L214F/R277K/K476N mutations into the p6HRT-PROT prokaryotic expression vector. We also generated 2 additional constructs that either lacked either the L74V mutation (SGS3 Δ L74V) or the K476N mutation (SGS3 Δ K476N). Each of the mutant enzymes was purified to homogeneity and analyzed for DNA-dependent DNA polymerase activity using either 0.1 μ M or 1.0 μ M dNTP. The DNA polymerization activity of all three mutant RTs was compromised compared to the WT enzyme, Figure 21. Specifically, the mutant RTs generated less full-length DNA product and there was an increase in the accumulation of shorter DNA products compared to the WT enzyme. We also assessed DNA polymerization by SGS3 and WT HIV-1 RT under processive conditions. The results in Figure 22 show that SGS3 RT is less processive than the WT enzyme at low (1.0 μ M) and high (10.0 μ M) concentrations of dNTP.

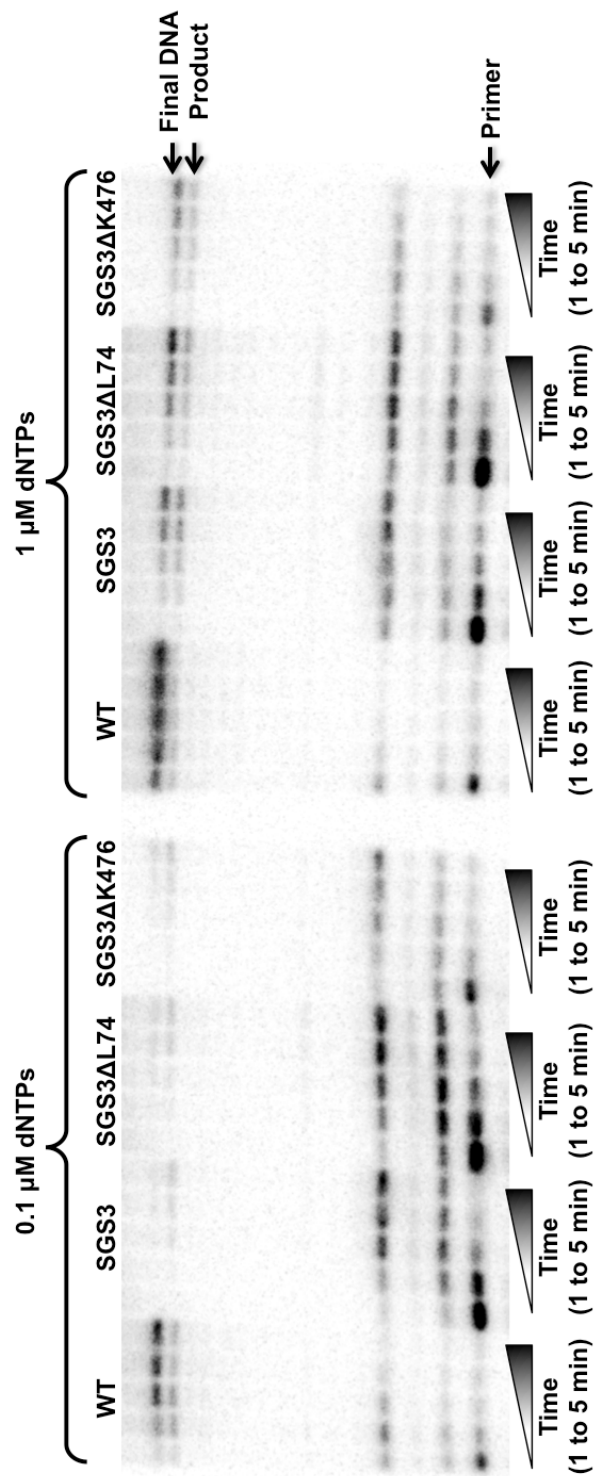


Figure 21: DNA-dependent DNA polymerase activities of WT, SGS3, SGS3ΔL74V and SGS3ΔK476N HIV-1 RT

Representative autoradiogram of the DNA-dependent DNA polymerase activity of WT, SGS3, SGS3ΔL74V and SGS3ΔK476N HIV-1 RT under steady-state assay conditions. Reactions were carried out at 0.1 and 1.0 μM dNTP. Reactions times were 0, 1, 2, 3, 4 and 5 min.

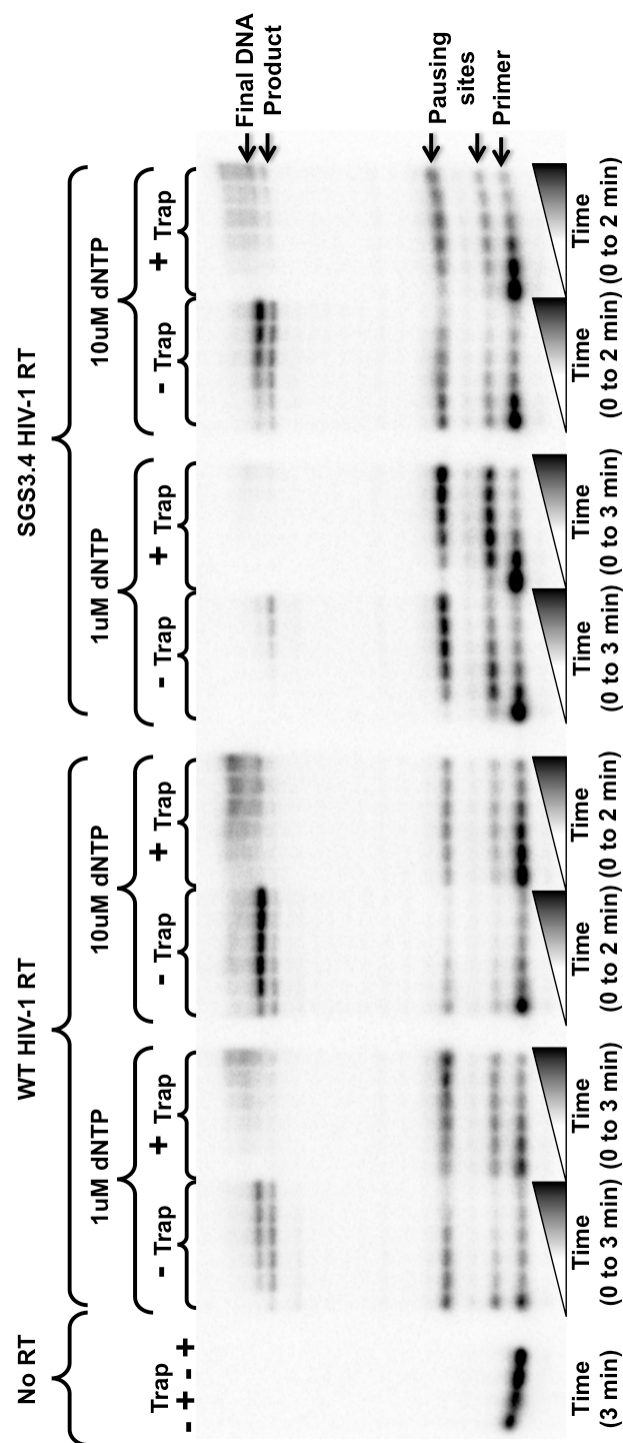


Figure 22: DNA-dependent DNA polymerase activities of WT and mutant HIV-1 RT under processive conditions

Representative autoradiogram of the DNA-dependent DNA polymerase activity of WT, SGS3, SGS3 Δ L74V and SGS3 Δ K476N HIV-1 RT under processive steady-state assay conditions. Reactions were carried out at 1.0 and 10 μ M dNTP. Unlabeled T36/P19 T/P was used as a trap at 200 nM. Reaction times were 0, 30, 60, 90, 120 and 180 sec for 1.0 μ M dNTP or 0, 15, 30, 60, 90 and 120 sec for 10 μ M dNTP.

4.5.2 3'-azido-ddGTP incorporation and 3'-azido-ddGMP excision activity of WT and mutant HIV-1 RT

During HIV-1 replication there are multiple opportunities for RT to incorporate and excise nucleotide analogs. As such, we initially assessed the ability of WT and mutant HIV-1 RT to synthesize full-length DNA product in the presence of 3'-azido-ddGTP and ATP under steady-state assay conditions. Figure 23 shows that in the presence of 5 μ M 3'-azido-ddGTP and 3 mM ATP, the SGS3 and SGS3 Δ K476N RTs synthesized significantly greater amounts of full-length DNA than did the WT or SGS3 Δ L74V enzymes. This increase in DNA product formation by these RTs appeared to be driven by a decrease in the frequency of 3'-azido-ddGMP chain-termination. Of note, this assay was carried-out over a long time period (5-90 min) to allow for the enzyme to excise the chain-terminating 3'-azido-ddGMP moiety. In this regard, there was no evidence of a decrease in chain-termination through excision at any of the sites at which 3'-azido-ddGTP had been incorporated. Taken together, this data suggested that 3'-azido-ddG resistance was driven by a discrimination phenotype mediated by the L74V mutation.

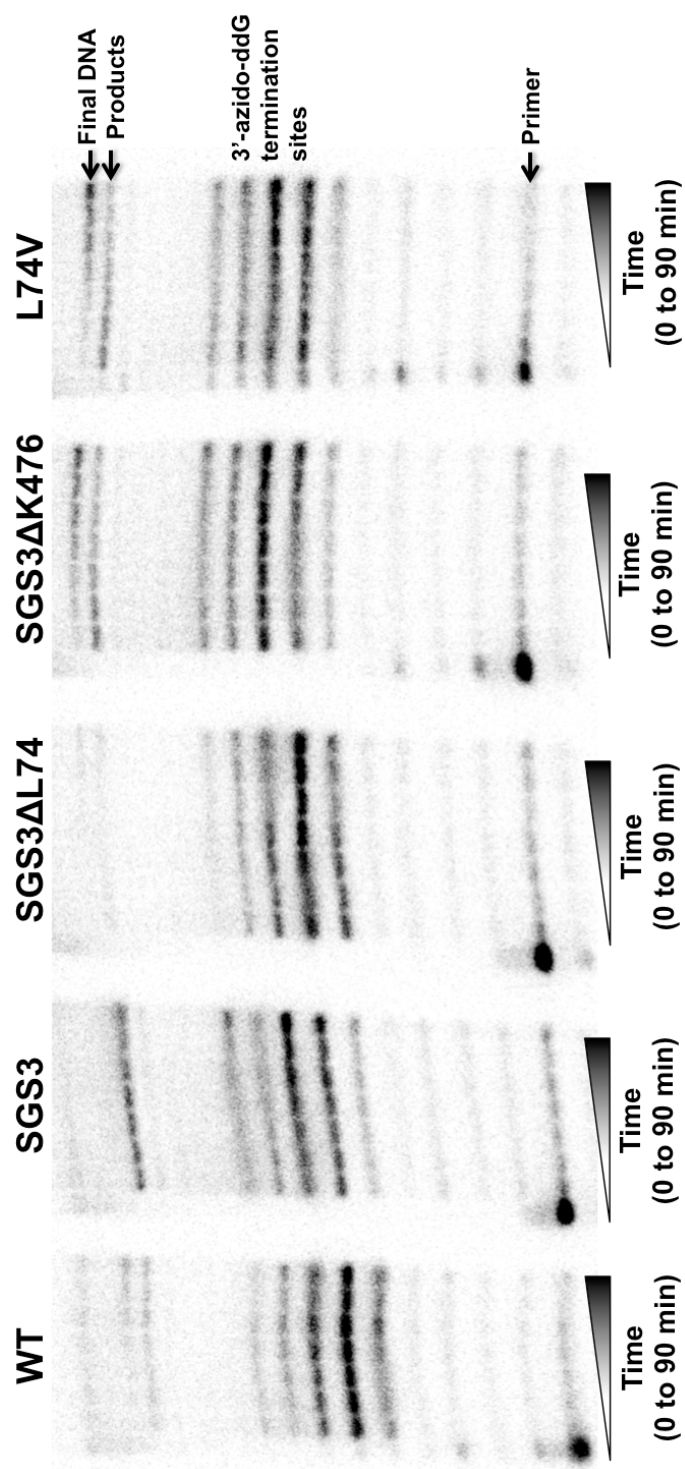


Figure 23: 3'-Azido-ddGTP incorporation and 3'-azido-ddGMP excision activity

Representative autoradiogram of the DNA-dependent DNA polymerase activity of WT, SGS3, SGS3 Δ L74V and SGS3 Δ K476N HIV-1 RT in the presence of 5 μ M 3'-azido-ddGTP and 3 mM ATP under steady-state assay conditions. Reaction times were 0, 5, 10, 15, 30, 45, 60 and 90 min.

4.5.3 Pre-steady-state incorporation of dGTP and 3'-azido-ddGTP by WT and mutant HIV-1 RT

Pre-steady state kinetic analyses were carried out to elucidate the interactions of dGTP and 3'-azido-ddGTP with the polymerase active sites of WT and SGS3 HIV-1 RT, Table 10. These experiments defined the maximum rates of nucleotide incorporation (k_{pol}), the nucleotide dissociation constants (K_d), and the catalytic efficiencies of incorporation (k_{pol}/K_d). The k_{pol}/K_d values for the incorporation of dGTP by WT or SGS3 HIV-1 RT were essentially identical, suggesting that the L74V/F77L/V106I/L214F/R277K/K476N mutations do not adversely affect single nucleotide turn-over events. The selectivity of RT, which is defined as $(k_{pol}/K_d)^{dGTP} / (k_{pol}/K_d)^{3'-azido-ddGTP}$, is an indication of the ability of the WT or SGS RT to discriminate between dGTP and 3'-azido-ddGTP. As reported previously, the WT enzyme cannot discriminate between dGTP and 3'-azido-ddGTP (selectivity < 1) [337,344]. By contrast, the mutations in SGS3 RT independently increased the selectivity of the enzyme for the natural substrate *versus* 3'-azido-ddGTP, Table 10. The observed 3'-azido-ddGTP resistance of SGS3 RT could primarily be attributed to a decrease in K_d and not a decrease in k_{pol} . Unfortunately, we were unable to purify sufficient quantities of the SGS3 Δ L74V and SGS3 Δ K476N RTs to perform pre-steady-state kinetic assays. Therefore, we also carried out analyses to elucidate the interactions of dGTP and 3'-azido-ddGTP with the polymerase active site of L74V HIV-1 RT, Table 10. Similar to SGS3 RT, the L74V enzyme could effectively discriminate between dGTP and 3'-azido-ddGTP by decreasing the affinity of the nucleotide analog for DNA polymerase active site. Of note, the calculated 3'-azido-ddGTP fold-resistance (Fold-R) values for SGS3 and L74V RT were similar.

Table 10: Pre-steady-state kinetic values for incorporation of dGTP and 3'-azido-2',3'-ddG

Nucleotide	k_{pol} (s^{-1})	K_d (μM)	k_{pol}/k_d ($\mu M^{-1}s^{-1}$)	^a Selectivity	^b Fold-R
<i>WT HIV-1 RT</i>					
dGTP	^c 18.1 ± 6.7	1.3 ± 1.2	13.98	-	
3'-azido-ddGTP	18.2 ± 7.6	0.7 ± 0.1	26.47	0.53	1
<i>SGS3 HIV-1 RT</i>					
dGTP	20.1 ± 1.8	1.8 ± 0.9	11.2	-	
3'-azido-ddGTP	20.0 ± 4.6	3.6 ± 1.7	5.6	1.99	3.75
<i>L74V HIV-1 RT</i>					
dGTP	21.5 ± 11.6	0.6 ± 0.3	38.49	-	
3'-azido-ddGTP	28.2 ± 11.3	1.2 ± 0.6	23.57	1.63	3.1

^a Selectivity is $(k_{pol}/K_d)^{dGTP}/(k_{pol}/K_d)^{3'-azido-ddGTP}$.

^b Resistance (*n*-fold) is selectivity^{mutant}/selectivity^{WT}.

^c Data are the mean \pm S.D. determined from at least three independent experiments.

4.5.4 Excision of 3'-azido-ddGMP by WT and mutant HIV-1 RT

Prior studies have shown that the L74V mutation significantly attenuates RTs ability to excise the chain-terminating NRTI-MP [239,369]. However, we recently demonstrated that the N348I mutation in the connection domain of RT could augment the excision activity of the L74V enzyme by selectively decreasing the frequency of secondary RNase H cleavages that reduce the overall efficiency of the excision reaction [370]. Therefore, we next examined the ability of WT, SGS3, SGS3 Δ L74V and SGS3 Δ K476N HIV-1 RT to excise 3'-azido-ddGMP and rescue DNA synthesis from chain-terminated DNA/DNA and RNA/DNA T/Ps, Figure 24. An excision-competent RT containing the TAMs D67N/K70R/T215F/K219Q (AZT^R) was also included as a control in these experiments. On the DNA/DNA T/P substrate, the SGS3 and SGS3 Δ K476N RTs were significantly less efficient in excising 3'-azido-ddGMP than was the WT enzyme. The ATP-mediated excision activity of the SGS3 Δ L74V RT was similar to that of the WT enzyme.

These observations are consistent with the L74V mutation significantly reducing RTs ability to excise chain-terminating nucleotide analogs. In contrast, on the RNA/DNA T/P substrate, the ATP-mediated excision activity of SGS3 RT was comparable to that of the WT enzyme. Reversion of L74V to the WT codon (i.e. SGS3 Δ L74V) significantly increased the enzyme's ability to excise 3'-azido-ddGMP suggesting that the F77L/V106I/L214F/R277K/K476N mutations contribute to an excision phenotype on an RNA/DNA, but not DNA/DNA, T/P. Reversion of K476N to the WT codon (i.e. SGS3 Δ K476N RT) almost completely abolished RTs ability to excise 3'-azido-ddGMP indicating that the K476N mutation counteracts the negative effect of L74 on excision.

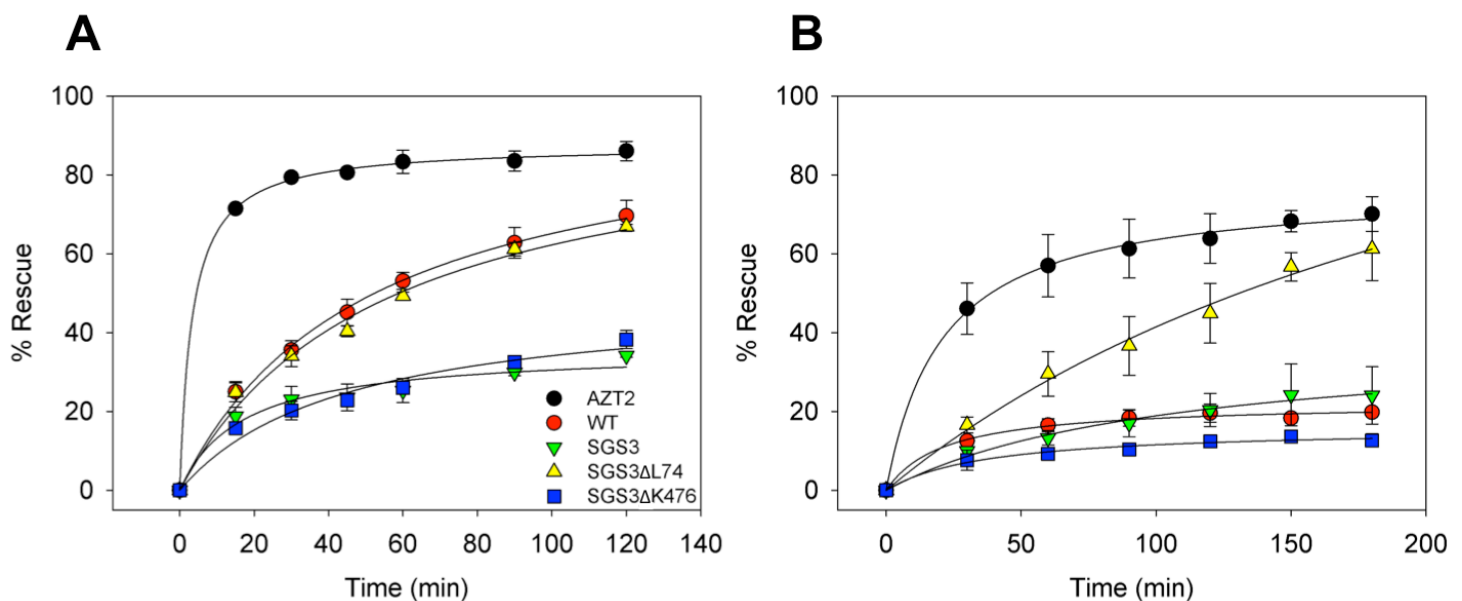


Figure 24: ATP-mediated excision of 3'-azido-ddGMP and rescue of DNA synthesis by HIV-1 RT on chain terminated T/P

Reaction performed using T/Ps with the same sequence. A. DNA/DNA T/P substrate and B. RNA/DNA T/P substrate. Data are the mean \pm standard deviation determined from at least 3 independent experiments.

We previously delineated the relationship between AZT-MP excision efficiency and RNase H activity on a RNA/DNA T/P substrate that was essentially identical to the one used in these experiments [340,343]. These studies showed that the primary polymerase-dependent RNase H cleavages do not impact the enzyme's excision efficiency, but polymerase-independent RNase H cleavages that reduce the RNA/DNA duplex length to less than 12 nucleotides abolish the excision activity. In light of this, we next evaluated the RNase H activity of the WT and mutant RTs that occurred during the ATP-mediated excision reactions. The data in Figure.25 shows that the SGS3 and SGS3 Δ L74V RTs carryout less secondary RNase H cleavages. As a result, there is prolonged preservation of T/P substrates with duplex lengths of 15-18 nucleotides. As described above, RT can efficiently excise a chain-terminating NRTI-MP from these substrates [340,343]. In contrast, these T/P substrates (with duplex lengths of 15-18 nucleotides) are not preserved in reactions carried out by the WT and SGS3 Δ K476N RTs.

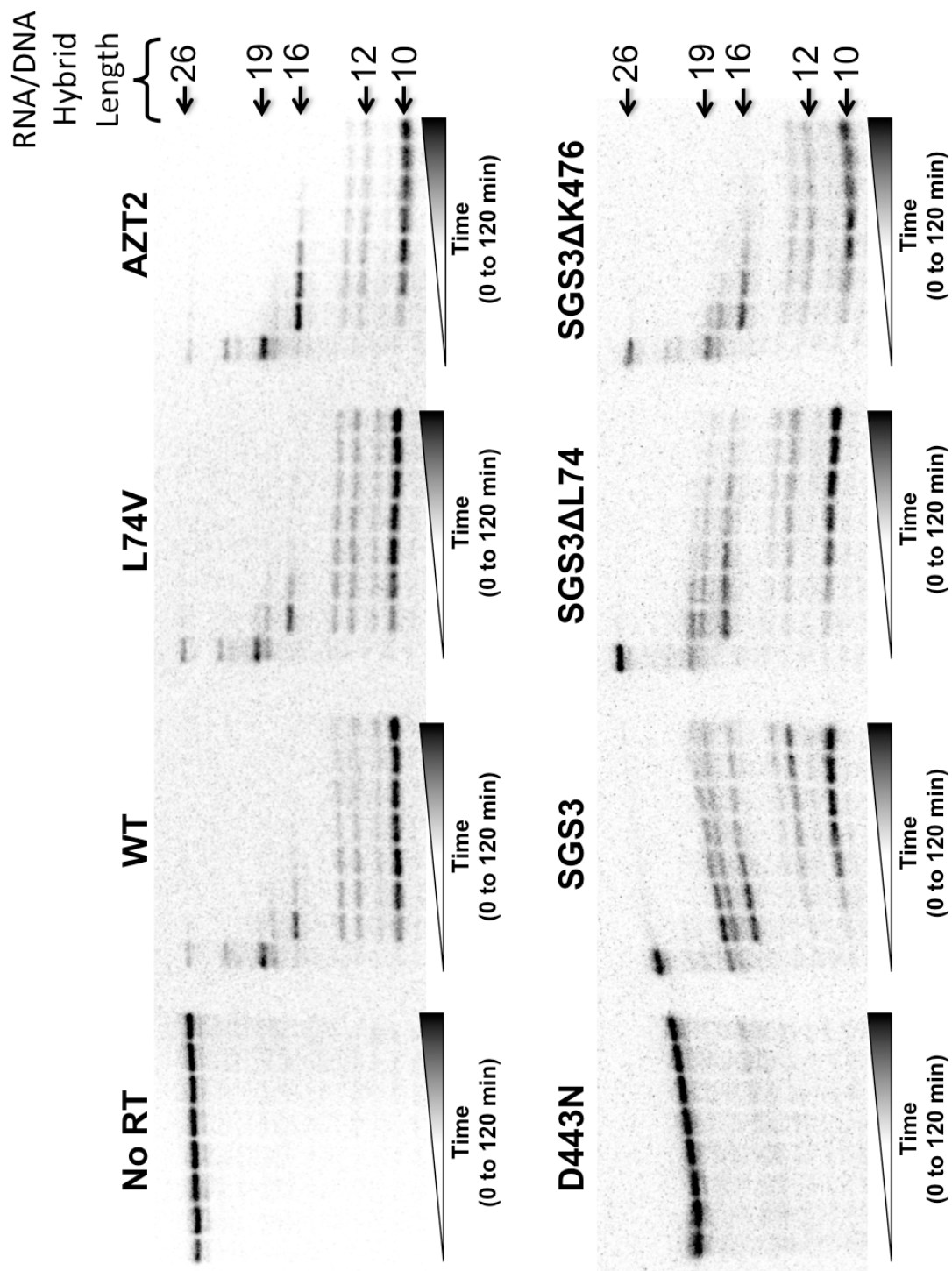


Figure 25: Representative autoradiogram of the RNase H cleavage activity that occurs during the 3'-azido-ddGMP excision reaction

No RNA degradation was observed in the absence of RT or for RNase H null D443N. The reaction times were 10, 5 15, 30, 45, 60, 90 and 120 min.

4.6 DISCUSSION

In this study we show that the L74V/F77L/V106I/L214F/R277K/K476N mutations in HIV-1 RT confer 3'-azido-ddG resistance primarily through discrimination with some contribution from excision. The NRTI-TP discrimination resistance phenotype was mediated by the L74V mutation in HIV-1 RT, whereas the NRTI-MP excision phenotype was primarily mediated by the K476N mutation. We were unable to clarify the relative contributions of the F77L, V106I, L214F and R277K in HIV-1 RT to 3'-azido-ddG resistance.

Our kinetic data shows that the L74V mutation allows HIV-1 RT to effectively discriminate between dGTP and 3'-azido-ddGTP by selectively decreasing the nucleotide analog's binding affinity (i.e. K_d). L74V is selected by other purine analogs including didanosine and abacavir [242,371], and a prior pre-steady-state kinetic study reported that the L74V mutation allows RT to discriminate between dATP and ddATP [372]. Interestingly, L74V in HIV-1 RT confers ~ 3-fold resistance to 3'-azido-ddGTP (Table 10), but HIV-1 containing the L74V mutation remains sensitive to inhibition by 3'-azido-ddG [344]. In this regard, it has been shown that the K65R mutation in HIV-1 RT allows the enzyme to discriminate between TTP and AZT-TP [366,373]. Like L74V, K65R significantly decreases the ATP-mediated excision activity of the enzyme, and it was proposed that the combination of these opposing mechanisms results in the increased susceptibility of HIV-1 containing the K65R mutation to AZT [373]. In this study, we show that HIV-1 has compensated for the decreased excision activity of L74V RT through the gain of additional mutations, particularly K476N, that augments the enzyme's ability unblock the chain-terminating 3'-azido-ddGMP moiety on RNA/DNA T/P substrates. Indeed, we found that K476N restored the enzyme's ability to excise 3'-azido-ddGMP on an RNA/DNA, but not DNA/DNA, T/P by selectively decreasing the frequency of secondary RNase H cleavage

events that preserved excision-competent RNA/DNA T/P substrates with duplex lengths ranging from 15-18 nucleotides. Taken together, these findings suggest that K476N, like the A360V, N348I and Q509L mutations, impacts the efficiency of the excision reaction by an RNase H-dependent mechanism [340,352,355,374].

In Figure 21, we show that SGS3 RT has reduced DNA polymerase activity compared to the WT enzyme. However, the pre-steady-state kinetic analyses demonstrated that the catalytic efficiency ratios (i.e. k_{pol}/K_d) for SGS3 and WT HIV-1 RT were comparable. These data suggest that the L74V/F77L/V106I/L214F/R277K/K476N mutations do not directly impact the DNA polymerase active site of HIV-1 RT. Instead, we show that the decrease in DNA polymerase activity of SGS3 RT under steady-state assay conditions is likely due to decreased processivity, Figure 22. A decrease in the *in vitro* processivity of L74V HIV-1 RT has been documented previously [372,375].

The level of resistance that HIV-1 achieves to 3'-azido-ddG after *in vitro* selection is modest (3- to 4-fold) and requires multiple resistance mutations, including L74V that markedly reduce nucleotide excision. This L74V-mediated reduction in excision was only partially reversed by K476N in the RNase H domain, suggesting that there are molecular constraints on HIV-1 RT such that it does not readily evolve high-level resistance to 3'-azido-ddG through discrimination and excision mechanisms, either alone or in combination. The data presented in this report provide genetic and biochemical insights into the favorable activity profile of 3'-azido-ddG against NRTI-resistant virus.

In conclusion, our analyses reveal that HIV-1 resistance to 3'-azido-ddG is mediated by both the NRTI-TP discrimination and NRTI-MP excision phenotypes. By comparison, AZT selects for TAMs in HIV-1 that confer resistance exclusively via the NRTI-MP excision

phenotype. As such, these data strongly reinforce the thesis that the nucleoside base structure is major determinant of HIV-1 resistance to the 3'-azido-2',3'-dideoxynucleosides and that further optimization of base structure is possible to enhance the activity and resistance profile of 3'-azido-2',3'-dideoxynucleosides.

5.0 FINAL SUMMARY AND FUTURE DIRECTIONS

There are limitations of the currently available NRTI including development of resistance, cross-resistance between inhibitors and high levels of toxicity. These issues restrict the usefulness of NRTI in HIV-1 therapy. This is compounded by a gap in knowledge for the rationale development of new NRTI inhibitors. Specifically, it is not known what structural components of NRTI are the determining factors for activity, resistance and toxicity. This thesis aims to delineate these structure-activity-resistance relationships using 3'-azido-2',3'-dideoxynucleoside analogs.

This thesis adds to the field of drug resistance, but a greater understanding of these observations in the context of current HIV-1 therapy is necessary to evaluate the selected resistance and mechanisms by which 3'-azido-2',3'-dideoxynucleosides inhibit HIV-1 RT in order to develop novel and potent antiretrovirals.

5.1 SUMMARY OF 3'-AZIDO-2',3'-DIDEOXYNUCLEOSIDE-SELECTED RESISTANCE IN HIV-1

We have described that NRTIs with different base structures, but the same 3'-azido-2',3'-dideoxyribose sugar, selected for divergent resistance mutations in RT *in vitro*. In addition, 3'-azido-2',3'-ddG was only able to select a very low level of resistance while maintaining activity

against TAM containing viruses. Not surprisingly, 3'-azido-ddG did not select for TAMs, but instead a group of mutations unknown to be selected by any other FDA-approved compound. Furthermore, it was discovered that 3'-azido-ddG selected for a diverse population of mutants with different mutations determined by single genome sequencing. Conversely, 3'-azido-ddC selected the single RT mutation V75I and 3'-azido-ddA selected no RT mutations. Interestingly, the EC₅₀ value of 3'-azido-ddA did not change during the course of the selection, but nevertheless virus was able to replicate through increasingly high concentrations of inhibitor. We were unable to determine the mechanism of this 3'-azido-ddA-resistance phenotype.

These findings support the hypothesis that the base component of NRTIs are important factors in determining their antiretroviral activity and suggests that NRTI base structures, particularly guanosine, could be modified to alter the mechanisms and genetic barriers to HIV-1 drug resistance. These insights should guide the synthesis of novel NRTIs that pose a high genetic barrier to HIV-1 resistance and are active against HIV-1 RT harboring drug-resistance mutations to other inhibitors.

5.2 SUMMARY OF BIOCHEMICAL MECHANISMS OF RESISTANCE TO 3'-AZIDO-ddG BY HIV-1

The molecular mechanism of 3'-azido-ddG-resistance by selected mutations was determined biochemically. Interestingly, HIV-1 only achieves a modest level of resistance to 3'-azido-ddG after extensive *in vitro* selection and requires the accumulation of multiple resistance mutations, including L74V that markedly reduces nucleotide excision activity. This L74V-mediated reduction in excision was only partial reversed by K476N in the RNase H domain,

suggesting that there are molecular constraints on HIV-1 RT such that it does not readily evolve high-level resistance to 3'-azido-ddG through discrimination and excision mechanisms, either alone or in combination. We have presented genetic and biochemical insights into the favorable activity profile of 3'-azido-ddG against NRTI-resistant virus.

In conclusion, our analyses reveal that HIV-1 resistance to 3'-azido-ddG is mediated by a dual mechanism with both NRTI-TP discrimination and NRTI-MP excision phenotypes. By comparison, the thymine analog AZT selects for TAMs in HIV-1 RT that confer resistance exclusively through the NRTI-MP excision phenotype. These data strongly reinforce the hypothesis that the nucleoside base structure is major determinant of HIV-1 resistance to the 3'-azido-2',3'-dideoxynucleosides and that further optimization of base structure is possible to enhance the activity and resistance profile of 3'-azido-2',3'-dideoxynucleosides.

5.3 FUTURE DIRECTIONS OF 3'-AZIDO-2',3'-DIDEOXYNUCLEOSIDE ANTIRETROVIRAL RESEARCH

5.3.1 Limitations of *in vitro* selections and additional experiments

A major limitation of the *in vitro* selection experiments described in Chapter 2 is that each was only performed once, $n = 1$, due to the length of time required for each selection and the cost and space required. In retrospect, performing selections in parallel would have been more ideal. It is currently not known if different mutation patterns could promote additional resistance to these 3'-azido-2',3'-dideoxynucleoside analog inhibitors. It is highly suggested that these experiments be repeated to determine other possible outcomes. Additionally, it would be prudent to also

passage virus other than wild-type to determine how the input virus affects the selected resistance. Key mutations, such as L74V, could be studied in depth using this method. Additionally, it would be interesting to determine the reversion of mutations following passage in the absence of inhibitor. Do the mutations simply disappear over time, or are they maintained over many viral replications?

Another concern is the level of inhibitor present in media and cells over time. The current protocol used here likely has a time dependent decrease in concentration of inhibitor. This drop in inhibitor concentration allows some low level of viral replication to occur, which is important to allow the virus to accumulate random mutations and evolve resistance phenotypes.

The process of selecting resistant virus described here is tedious and stochastic. It would be interesting to initiate a selection by starting with a diverse population of mutations instead of a clonal virus. Susceptible viruses would immediately be blocked from replicating and would be quickly eliminated. Resistant viruses would be able to become dominant quickly because their resistance mutations would already be preexisting. The key to the success of this experiment of rapid selection is creating a controlled, random mutagenesis library of viruses to obtain all possible base substitutions isolated in the RT gene.

5.3.2 Modifications of base structure

We have provided evidence that the base structure is a determinant of antiviral activity. For example, 3'-azido-2',3'-dideoxypurine analogs are potent inhibitors of TAM containing RT. However, it is hypothesized that additional modifications of the natural purine base structures, Figure 26, could enhance activity, metabolism and cytotoxicity. There have been multiple studies

by our group and others that indicate that this rational drug design approach is an efficient method to convert antiretroviral lead compounds into potent inhibitors [376-383].

X	Y	Z	Comment	A	B	Base
CH	N	N	natural purine	NH ₂	H	adenine
				O	NH ₂	guanine
CH	CH	N	7-deaza	H, O, Br, Cl, F, Me, CN, NH ₂ OMe, NMe ₂ , etc.	modified base	
NH	NH	N	8-aza			
CH	N	CH	3-deaza			
CH	CF, CCl, CBr, CMe, CCN, CCONH ₂ , COH	N	7- modified			
CF, CCl, CN ₃ , CBr, COH	N	N	8- modified			

Figure 26: Matrix of proposed heterobase-modified 3'-azido-2',3'-dideoxypurine analogs

All NRTI are administered as prodrugs that must be metabolized into triphosphate forms to be active inhibitors. The first phosphorylation step is often rate limiting and can potentially be eliminated by administering the NRTI as an NRTI-MP, either as a phosphate or phosphonate, like TDF. This can increase the amount of intracellular active metabolite present and effectively enhance potency.

Of note, there are only three FDA-approved purine-base NRTI currently available: ddI, TDF and ABC. These inhibitors have greatly enhanced the antiretroviral arsenal available for combination HIV-1 therapy, but are plagued with several undesirable attributes. They are all vulnerable to selected resistance and cross-resistance, ABC is associated with a hypersensitivity

reaction in a small percentage of the population, and TDF is incompatible with ddI and the PI atazanavir [384-387]. The base-modified 3'-azido-2',3'-dideoxypurine analogs may be able to circumvent these problems and provide additional therapeutic compounds to combat an ever changing disease.

5.3.3 HIV-1 subtypes and antiretroviral inhibitors

There is a major discrepancy in most HIV-1 research because subtype B is the most prominent subtype in industrialized nations and used in most research studies, however subtype C is most prevalent globally [17]. The effects of current treatments on individuals infected with non-subtype B viruses are not clear. Some evidence suggests that the different subtypes may respond differently to FDA-approved therapeutics [388-390]. This is compounded by non-B subtype polymorphisms that are often observed as selected secondary resistance mutations in subtype B [388,391,392]. There are critical distinctions between the different subtypes that may require different antiretrovirals for effective therapeutics. For example, subtype D clinical isolates have been observed *in vitro* to have reduced drug sensitivity to AZT and 3TC due to its rapid growth kinetics unmatched by subtypes A, B, C and E [393]. Unfortunately, these NRTI are important in resource limiting settings where subtype D is most prevalent. These observations imply that the current laboratory standard HIV-1 subtype B may not be the most appropriate model for the study of all HIV-1 resistance development. Additionally, it would be advantageous to know the subtype before initiation of therapy to avoid known inhibitors with subtype-inherent resistance. However, the expense and complexity of genotyping restricts the practicality of this option and the limited availability of alternative regimens affords little variation in treatment choices based on genotyping results. There is a gap in the research of non-subtype B antiretrovirals that

highlights the need for the development of novel, potent inhibitors specifically directed for activity to towards these viruses.

5.3.4 HIV-1 resistance to 3'-azido-ddA

During *in vitro* selection experiments, we were able to passage virus through media containing increasing concentrations of 3'-azido-ddA well above the EC₅₀. However, there were no mutations selected in the gene of RT and accordingly there was no increase in observed EC₅₀ by the selected virus. There are several possibilities for this phenomenon. First, the 3'-azido-ddA resistance may be due to a mechanism in which the virus is able to efficiently infect cells that are not actively metabolizing the inhibitor into the triphosphate form. This may arise from the accumulation of mutations outside of RT, particularly in Env, that would increase the infectivity of the virus in order to increase the probability of infecting the small percentage of these cells. Additionally, even if cells are metabolizing the pro-drug into the active compound, it is possible that the intracellular distribution of the active inhibitor-triphosphate does not coincide with the physical location of the reverse transcriptase complex in the cytoplasm.

It will be critical to sequence the entire genome of this passaged virus to look for mutations outside of RT that could potentially contribute to this effect. Additionally, it would be advantageous to identify which cells are not metabolizing the drug and why. It is likely that during certain parts of the cell cycle, the cells are not expressing the kinases or phosphoryl transferases required to metabolize the triphosphate, and thus these particular cells are susceptible to infection.

5.3.5 Sequencing single-genomes of clinical isolates

The genotype of the virus has a direct impact on the resistance to antiretrovirals and many viral phenotypes can be inferred from sequences alone [394-397]. Our selection of 3'-azido-ddG-resistant HIV-1 resulted in a complex population of viruses with mixed genotypes [364]. Standard population sequencing of bulk virus was insufficient to sample the RT genome sequences because the linkage of mutations within each genome was not consistent. Single genome sequencing was required to determine the actual full-length genotypes of individual viral genomic RT. It would be interesting to perform this on patient isolates to determine the dynamics of resistance mutation selection *in vivo*.

APPENDIX A

A.1 SEQUENCE OF HIV-1_{xxLAI-MO} RT

The complete sequence of wild-type HIV-1_{xxLAI-MO} RT is provided in this appendix. The positions of the unique sites for restriction endonuclease cleavage by XmaI, MluI, XbaI and NgoMIV have been noted. Additionally, the codons of all major selected mutations in this thesis have been highlighted (yellow for 3'-azido-ddG-associated, blue for 3'-azido-ddC-associated) for reference. The forward sequencing primers used are marked in green.

XmaI																
	3	6	9	12	15	18	21	24	27	30	33	36	39	42	45	48
nt	CCC	ATT	AGT	CCT	ATT	GAA	ACT	GTA	CCA	GTA	AAA	TTA	AAG	CCC	GGG	ATG
AA	Pro	Ile	Ser	Pro	Ile	Glu	Thr	Val	Pro	Val	Lys	Leu	Lys	Pro	Gly	Met
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
	51	54	57	60	63	66	69	72	75	78	81	84	87	90	93	96
nt	GAT	GGC	CCA	AAA	GTT	AAA	CAA	TGG	CCA	TTG	ACA	GAA	GAA	AAA	ATA	AAA
AA	Asp	Gly	Pro	Lys	Val	Lys	Gln	Trp	Pro	Leu	Thr	Glu	Glu	Lys	Ile	Lys
	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32
	99	102	105	108	111	114	117	120	123	126	129	132	135	138	141	144
nt	GCA	TTA	GTA	GAA	ATT	TGT	ACA	GAA	ATG	GAA	AAG	GAA	GGG	AAA	ATT	TCA
AA	Ala	Leu	Val	Glu	Ile	Cys	Thr	Glu	Met	Glu	Lys	Glu	Gly	Lys	Ile	Ser
	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48
	147	150	153	156	159	162	165	168	171	174	177	180	183	186	189	192
nt	AAA	ATT	GGG	CCT	GAA	AAT	CCA	TAC	AAT	ACT	CCA	GTA	TTT	GCC	ATA	AAG
AA	Lys	Ile	Gly	Pro	Glu	Sn	Pro	Tyr	Ssn	Thr	Pro	Val	Phe	Ala	Ile	Lys
	49	50	51	52	53	54	55	56	57	58	59	60	61	62	63	64
	195	198	201	204	207	210	213	216	219	222	225	228	231	234	237	240
nt	AAA	AAA	GAC	AGT	ACT	AAA	TGG	AGA	AAA	TTA	GTA	GAT	TTC	AGA	GAA	CTT
AA	Lys	Lys	Asp	ser	Thr	Lys	Trp	Arg	Lys	Leu	Val	Asp	Phe	Arg	Glu	Leu
	65	66	67	68	69	70	71	72	73	74	75	76	77	78	79	80
	243	246	249	252	255	258	261	264	267	270	273	276	279	282	285	288
nt	AAT	AAG	AGA	ACT	CAA	GAC	TTC	TGG	GAA	GTT	CAA	TTA	GGA	ATA	CCA	CAT
AA	Asn	Lys	Arg	Thr	Gln	Asp	Phe	Trp	Glu	Val	Gln	Leu	Gly	Ile	Pro	His
	81	82	83	84	85	86	87	88	89	90	91	92	93	94	95	96
	291	294	297	300	303	306	309	312	315	318	321	324	327	330	333	336
nt	CCC	GCA	GGG	TTA	AAA	AAG	AAA	AAA	TCA	GTA	ACA	GTA	CTG	GAT	GTG	GGT
AA	Pro	Ala	gly	Leu	Lys	Lys	Lys	Lys	Ser	Val	Thr	Val	Leu	Asp	Val	Gly
	97	98	99	100	101	102	103	104	105	106	107	108	109	110	111	112
	339	342	345	348	351	354	357	360	363	366	369	372	375	378	381	384
nt	GAT	GCA	TAT	TTT	TCA	GTT	CCC	TTA	GAT	GAA	GAC	TTC	AGG	AAG	TAT	ACT
AA	Asp	Ala	Tyr	Phe	Ser	Val	Pro	Leu	Asp	Glu	Asp	Phe	Arg	Lys	Tyr	Thr
	113	114	115	116	117	118	119	120	121	122	123	124	125	126	127	128
	387	390	393	396	399	402	405	408	411	414	417	420	423	426	429	432
nt	GCA	TTT	ACC	ATA	CCT	AGT	ATA	AAC	AAT	GAG	ACA	CCA	GGG	ATT	AGA	TAT
AA	Ala	Phe	Thr	Ile	Pro	Ser	Ile	Asn	Asn	Glu	Thr	Pro	Gly	Ile	Arg	Tyr
	129	130	131	132	133	134	135	136	137	138	139	140	141	142	143	144
	435	438	441	444	447	450	453	456	459	462	465	468	471	474	477	480
nt	CAG	TAC	AAT	GTG	CTT	CCA	CAG	GGA	TGG	AAA	GGA	TCA	CCA	GCA	ATA	TTC
AA	Gln	Tyr	Asn	Val	Leu	Pro	Gln	Gly	Trp	Lys	Gly	Ser	Pro	Ala	Ile	Phe
	145	146	147	148	149	150	151	152	153	154	155	156	157	158	159	160
	483	486	489	492	495	498	501	504	507	510	513	516	519	522	525	528
nt	CAA	AGT	AGC	ATG	ACA	AAA	ATC	TTA	GAG	CCT	TTT	AGA	AAA	CAA	AAT	CCA
AA	Gln	Ser	Ser	Met	Thr	Lys	Ile	Leu	Glu	Pro	Phe	Arg	Lys	Gln	Asn	Pro
	161	162	163	164	165	166	167	168	169	170	171	172	173	174	175	176
	531	534	537	540	543	546	549	552	555	558	561	564	567	570	573	576
nt	GAC	ATA	GTT	ATC	TAT	CAA	TAC	ATG	GAT	GAT	TTG	TAT	GTA	GGA	TCT	GAC
AA	Asp	Ile	Val	Ile	Tyr	Gln	Try	Met	Asp	Asp	Leu	Try	Val	Gly	Ser	Asp
	177	178	179	180	181	182	183	184	185	186	187	188	189	190	191	192

nt AA	579	582	585	588	591	594	597	600	603	606	609	612	615	618	621	624
	TTA	GAA	ATA	GGG	CAG	CAT	AGA	ACA	AAA	ATA	GAG	GAG	CTG	AGA	CAA	CAT
	Leu	Glu	Ile	Gly	Gln	His	Arg	Thr	Lys	Ile	Glu	Glu	Leu	Arg	Gln	His
nt AA	193	194	195	196	197	198	199	200	201	202	203	204	205	206	207	208
	627	630	633	636	639	642	645	648	651	654	657	660	663	666	669	672
	CTG	TTG	AGG	TGG	GGA	CTT	ACC	ACA	CCA	GAC	AAA	AAA	CAT	CAG	AAA	GAA
nt AA	209	210	211	212	213	214	215	216	217	218	219	220	221	222	223	224
	Leu	Leu	Arg	Trp	Gly	Leu	Thr	Thr	Pro	Asp	Lys	Lys	His	Gln	Lys	Glu
	209	210	211	212	213	214	215	216	217	218	219	220	221	222	223	224
nt AA	675	678	681	684	687	690	693	696	699	702	705	708	711	714	717	720
	CCT	CCA	TTC	CTT	TGG	ATG	GGT	TAT	GAA	CTC	CAT	CCT	GAT	AAA	TGG	ACA
	Pro	Pro	Phe	Leu	Trp	Met	Gly	Tyr	Glu	Leu	His	Pro	Asp	Lys	Trp	Thr
nt AA	225	226	227	228	229	230	231	232	233	234	235	236	237	238	239	240
	723	726	729	732	735	738	741	744	747	750	753	756	759	762	765	768
	GTA	CAG	CCT	ATA	GTG	CTG	CCA	GAA	AAA	GAC	AGC	TGG	ACT	GTC	AAT	GAC
nt AA	241	242	243	244	245	246	247	248	249	250	251	252	253	254	255	256
	Val	Gln	Pro	Ile	Val	Leu	Pro	Glu	Lys	Asp	Ser	Trp	Thr	Val	Asn	asp
	241	242	243	244	245	246	247	248	249	250	251	252	253	254	255	256
nt AA	771	774	777	780	783	786	789	792	795	798	801	804	807	810	813	816
	ATA	CAG	AAG	TTA	GTG	GGA	AAA	TTG	AAT	TGG	GCA	AGT	CAG	ATT	TAC	CCA
	Ile	Gln	Lys	Leu	Val	Gly	Lys	Leu	Asn	Trp	Ala	Ser	Gln	Ile	Try	Pro
nt AA	257	258	259	260	261	262	263	264	265	266	267	268	269	270	271	272
	819	822	825	828	831	834	837	840	843	846	849	852	855	858	861	864
	GGG	ATT	AAA	GTA	AGG	CAA	TTA	TGT	AAA	CTC	CTT	AGA	GGA	ACC	AAA	GCA
nt AA	273	274	275	276	277	278	279	280	281	282	283	284	285	286	287	288
	Gly	Ile	Lys	Val	Arg	Gln	Leu	Cys	Lys	Leu	Leu	Arg	Gly	Thr	Lys	Ala
	273	274	275	276	277	278	279	280	281	282	283	284	285	286	287	288
nt AA	867	870	873	876	879	882	885	888	891	894	897	900	903	906	909	912
	CTA	ACA	GAA	GTA	ATA	CCA	CTA	ACA	GAA	GAA	GCA	GAG	CTA	GAA	CTG	GCA
	Leu	Thr	Glu	Val	Ile	Pro	Leu	Thr	Glu	Glu	Ala	Glu	Leu	Glu	Leu	Ala
nt AA	289	290	291	292	293	294	295	296	297	298	299	300	301	302	303	304
	915	918	921	924	927	930	933	936	939	942	945	948	951	954	957	960
	GAA	AAC	AGA	GAG	ATT	CTA	AAA	GAA	CCA	GTA	CAT	GGA	GTG	TAT	TAT	GAC
nt AA	305	306	307	308	309	310	311	312	313	314	315	316	317	318	319	320
	Glu	Asn	Arg	Glu	Ile	Leu	Lys	Glu	Pro	Val	His	Gly	Val	Tyr	Tyr	Asp
	305	306	307	308	309	310	311	312	313	314	315	316	317	318	319	320
nt AA	963	966	969	972	975	978	981	984	987	990	993	996	999	1002	1005	1008
	CCA	TCA	AAA	GAC	TTA	ATA	GCA	GAA	ATA	CAG	AAG	CAG	GGG	CAA	GGC	CAA
	Pro	Ser	Lys	Asp	Leu	Ile	Ala	Glu	Ile	Gln	Lys	Gln	Gly	Gln	Gly	Gln
nt AA	321	322	323	324	325	326	327	328	329	330	331	332	333	334	335	336
	1011	1014	1017	1020	1023	1026	1029	1032	1035	1038	1041	1044	1047	1050	1053	1056
	TGG	ACA	TAT	CAA	ATT	TAT	CAA	GAG	CCA	TTT	AAA	AAT	CTG	AAA	ACA	GGA
nt AA	337	338	339	340	341	342	343	344	345	346	347	348	349	350	351	352
	Trp	Thr	Tyr	Gln	Ile	Tyr	Gln	Glu	Pro	Phe	Lys	Asn	Leu	Lys	Thr	Gly
	337	338	339	340	341	342	343	344	345	346	347	348	349	350	351	352
MluI																
nt AA	1059	1062	1065	1068	1071	1074	1077	1080	1083	1086	1089	1092	1095	1098	1101	1104
	AAA	TAT	GCA	AGA	ACG	CGT	GGT	GCC	CAC	ACT	AAT	GAT	GTA	AAA	CAA	TTA
	Lys	Tyr	Ala	Arg	Thr	Arg	Gly	Ala	His	Thr	Asn	Asp	Val	Lys	Gln	Leu
nt AA	353	354	355	356	357	358	359	360	361	362	363	364	365	366	367	368
	1107	1110	1113	1116	1119	1122	1125	1128	1131	1134	1137	1140	1143	1146	1149	1152
	ACA	GAG	GCA	GTG	CAA	AAA	ATA	ACC	ACA	GAA	AGC	ATA	GTA	ATA	TGG	GGA
nt AA	369	370	371	372	373	374	375	376	377	378	379	380	381	382	383	384
	Thr	Glu	Ala	Val	Gln	Lys	Ile	Thr	Thr	Glu	Ser	Ile	Val	Ile	Trp	Gly
	369	370	371	372	373	374	375	376	377	378	379	380	381	382	383	384

	1155	1158	1161	1164	1167	1170	1173	1176	1179	1182	1185	1188	1191	1194	1197	1200
nt	AAG	ACT	CCT	AAA	TTT	AAA	CTA	CCC	ATA	CAA	AAG	GAA	ACA	TGG	GAA	ACA
AA	Lys	Thr	Pro	Lys	Phe	Lys	Leu	Pro	Ile	Gln	Lys	Glu	Thr	Trp	Glu	Thr
	385	386	387	388	389	390	391	392	393	394	395	396	397	398	399	400
	1203	1206	1209	1212	1215	1218	1221	1224	1227	1230	1233	1236	1239	1242	1245	1248
nt	TGG	TGG	ACA	GAG	TAT	TGG	CAA	GCC	ACC	TGG	ATT	CCT	GAG	TGG	GAG	TTT
AA	Trp	Trp	Thr	Glu	Tyr	Trp	Gln	Ala	Thr	Trp	Ile	Pro	Glu	Trp	Glu	Phe
	401	402	403	404	405	406	407	408	409	410	411	412	413	414	415	416
	1251	1254	1257	1260	1263	1266	1269	1272	1275	1278	1281	1284	1287	1290	1293	1296
nt	GTC	AAT	ACC	CCT	CCT	TTA	GTG	AAA	TTA	TGG	TAC	CAG	TTA	GAG	AAA	GAA
AA	Val	Asn	Thr	Pro	Pro	Leu	Val	Lys	Leu	Trp	Tyr	Gln	Leu	Glu	Lys	Glu
	417	418	419	420	421	422	423	424	425	426	427	428	429	430	431	432
	1299	1302	1305	1308	1311	1314	1317	1320	1323	1326	1329	1332	1335	1338	1341	1344
nt	CCC	ATA	GTA	GGA	GCA	GAA	ACG	TTC	TAT	GTA	GAT	GGG	GCA	GCT	AGC	AGG
AA	Pro	Ile	Val	Gly	Ala	Glu	Thr	Phe	Tyr	Val	Asp	Gly	Ala	Ala	Ser	Arg
	433	434	435	436	437	438	439	440	441	442	443	444	445	446	447	448
	1347	1350	1353	1356	1359	1362	1365	1368	1371	1374	1377	1380	1383	1386	1389	1392
nt	GAG	ACT	AAA	TTA	GGA	AAA	GCA	GGA	TAT	GTT	ACT	AAT	AGA	GGA	AGA	CAA
AA	Glu	Thr	Lys	Leu	Gly	Lys	Ala	Gly	Tyr	Val	Thr	Asn	Arg	Gly	Arg	Gln
	449	450	451	452	453	454	455	456	457	458	459	460	461	462	463	464
	1395	1398	1401	1404	1407	1410	1413	1416	1419	1422	1425	1428	1431	1434	1437	1440
nt	AAA	GTT	GTC	ACC	CTA	ACT	GAC	ACA	ACA	AAT	CAG	AAG	ACT	GAG	TTA	CAA
AA	Lys	Val	Val	Thr	Leu	Thr	Asp	Thr	Thr	Asn	Gln	Lys	Thr	Glu	Leu	Gln
	465	466	467	468	469	470	471	472	473	474	475	476	477	478	479	480
				RT8												
										XbaI						
	1443	1446	1449	1452	1455	1458	1461	1464	1467	1470	1473	1476	1479	1482	1485	1488
nt	GCA	ATT	CAT	CTA	GCT	TTG	CAG	GAT	TCG	GGT	CTA	GAA	GTA	AAT	ATA	GTA
AA	Ala	Ile	His	Leu	Ala	Leu	Gln	Asp	Ser	Gly	Leu	Glu	Val	Asn	Ile	Val
	481	482	483	484	485	486	487	488	489	490	491	492	493	494	495	496
	1491	1494	1497	1500	1503	1506	1509	1512	1515	1518	1521	1524	1527	1530	1533	1536
nt	ACA	GAC	TCA	CAA	TAT	GCA	TTA	GGA	ATC	ATT	CAA	GCA	CAA	CCA	GAT	AAA
AA	Thr	Asp	Ser	Gln	Tyr	Ala	Leu	Gly	Ile	Ile	Gln	Ala	Gln	Pro	Asp	Lys
	497	498	499	500	501	502	503	504	505	506	507	508	509	510	511	512
	1539	1542	1545	1548	1551	1554	1557	1560	1563	1566	1569	1572	1575	1578	1581	1584
nt	AGT	GAA	TCA	GAG	TTA	GTC	AAT	CAA	ATA	ATA	GAG	CAG	TTA	ATA	AAA	AAG
AA	Ser	Glu	Ser	Glu	Leu	Val	Asn	Gln	Ile	Ile	Glu	Gln	Leu	Ile	Lys	Lys
	513	514	515	516	517	518	519	520	521	522	523	524	525	526	527	528
	1587	1590	1593	1596	1599	1602	1605	1608	1611	1614	1617	1620	1623	1626	1629	1632
nt	GAA	AAG	GTC	TAT	CTG	GCA	TGG	GTA	CCA	GCA	CAC	AAA	GGA	ATT	GGA	GGA
AA	Glu	Lys	Val	Tyr	Leu	Ala	Trp	Val	Pro	Ala	His	Lys	Gly	Ile	Gly	Gly
	529	530	531	532	533	534	535	536	537	538	539	540	541	542	543	544
										NgmIV						
	1635	1638	1641	1644	1647	1650	1653	1656	1659	1662	1665	1668	1671	1674	1677	1680
nt	AAT	GAA	CAA	GTA	GAT	AAA	TTA	GTC	AGT	GCC	GGC	ATC	AGG	AAA	GTA	CTA
AA	Asn	Glu	Gln	Val	Asp	Lys	Leu	Val	Ser	Ala	Gly	Ile	Arg	Lys	Val	Leu
	545	546	547	548	549	550	551	552	553	554	555	556	557	558	559	560

APPENDIX B

B.1 NUCLEIC ACID OLIGOMERS

The studies described in this thesis required the use of many nucleic acid oligomers for molecular cloning, Sanger DNA sequencing, site-directed mutagenesis, substrate primer/templates, etc. All oligomers are described in Appendix B.

B.1.1 Site-Directed Mutagenesis Primers:

All RT site-directed mutants are designed and created from HIV-1_{xxLAI-MO}, Appendix A.

L74V

F: 5' -AGTACTAAATGGAGAAAAGTAGTAGATTTTCAGAGAACTTAATAAGAGAACTC-3'
R: 5' -GAGTTCTCTTATTAAGTTCTCTGAGATATACTACTTTTCTCCATTTAGTACT-3'

F77L

F: 5' -AGTACTAAATGGAGAAAATTAGTAGATCTCAGAGAACTTAATAAGAGAACTC-3'
R: 5' -GAGTTCTCTTATTAAGTTATCTGAGATCTACTACTTTTCTCCATTTAGTACT-3'

F77L/L74V

F: 5' -AGTACTAAATGGAGAAAAGTAGTAGATCTCAGAGAACTTAATAAGAGAACTC-3'
R: 5' -GAGTTCTCTTATTAAGTTCTCTGAGATCTACTACTTTTCTCCATTTAGTACT-3'

V75I

F: 5' -CATAAAGAAAAAGACAGTACTAAATGGAGAAAATTAATAGATTTTCAGAGAACTTAATAA-3'
R: 5' -TTATTAAGTTCTCTGAAATCTATTAATTTTCTCCATTTAGTACTGTCTTTTTTCTTTATG-3'

E122K

F: 5' -GTGATGCATATTTTTCAGTTCCCTTAGATAAAGACTTCAGGAAGTA-3'
R: 5' -TACTTCCTGAAGTCTTTATCTAAGGGAAGTGA AAAATATGCATCAC-3'

L214F

F: 5' -CTGTTGAGGTGGGGATTACCACACCAGACAAAAAAC-3'
R: 5' -GTTTTTTGTCTGGTGTGGTAAATCCCCACCTCAACAG-3'

N447S

F: 5' -CTATGTAGATGGGGCAGCTAGCAGGGAGACTAAATTA-3'
R: 5' -TAATTTAGTCTCCCTGCTAGCTGCCCCATCTACATAG-3'

K476N:

F: 5' -CCTAACTGACACAACAAATCAGAATACTGAGTTACAAGCAATTCATC-3'
R: 5' -GATGAATTGCTTGTAACCTCAGTATTCTGATTTGTTGTGTGTCAGTTAGG-3'

V518I

F: 5' -GCACAACCAGATAAAAAGTGAATCAGAGTTAATCAATCAAATAATAGAG-3'
R: 5' -CTCTATTATTTGATTGATTAACCTCTGATTCACTTTTATCTGGTTGTGC-3'

B.1.2 Reversion Site-Directed Mutagenesis Primers:

All RT single genome sequence clone isolates described below contained either of the following backgrounds in addition to the HIV-1_{xxLAI-MO} genotype, Appendix A:

SGS3.4 (L74V/F77L/V106I/L214F/R277K/K476N)

SGS1.2 (L74V/F77L/V106I/E122K/L214F/R277K/K447N/V518I/V531I)

SGS3.4ΔV74

F: 5' - CATAAAGAAAAAAGACAGTACTAAATGGAGAAAATTAGTAGATCTCAGAGAAC - 3'

R: 5' - GTTCTCTGAGATCTACTAATTTTCTCCATTTAGTACTGTCTTTTTTCTTTATG - 3'

SGS3.4ΔL77

F: 5' - GTACTAAATGGAGAAAAGTAGTAGATTTTCAGAGAACTTAATAAGAGAACTC - 3'

R: 5' - GAGTTCTCTTATTAAGTTCTCTGAAATCTACTACTTTTCTCCATTTAGTAC - 3'

SGS3.4ΔI106

F: 5' - CGCAGGGTTAAAAAAGAAAAAATCAGTAACAGTACTGGATGTGG - 3'

R: 5' - CCACATCCAGTACTGTTACTGATTTTTTCTTTTTTAACCTGCG - 3'

SGS3.4ΔF214

F: 5' - ACATCTGTTGAGGTGGGGATTAACACACCAGAC - 3'

R: 5' - GTCTGGTGTGGTTAATCCCCACCTCAACAGATGT - 3'

SGS3.4ΔR277

F: 5' - TCAGATTTACCCAGGGATTAAAGTAAGGCAATTATGTAAACTCCTT - 3'

R: 5' - AAGGAGTTTACATAATTGCCTTACTTTAATCCCTGGGTAAATCTGA - 3'

SGS3.4ΔN476

F: 5' - CTAAGTACACAACAAATCAGAAGACTGAGTTACAAGCAATTCAT - 3'

R: 5' - ATGAATTGCTTGTAAGTCTGATTTGTTGTGTCAGTTAG - 3'

SGS1.2ΔK122

F: 5' - TGGGTGATGCATATTTTTTCAGTTCCTTAGATGAGGACTTCAGGAAGTATAC - 3'

R: 5' - GTATACTTCCTGAAGTCCTCATCTAAGGGAAGTGAATAATATGCATCACCA - 3'

SGS1.2ΔN447

F: 5' - CTATGTAGATGGGGCAGCTAGCAGGGAGACTAAATTA - 3'

R: 5' - TAATTTAGTCTCCCTGCTAGCTGCCCCATCTACATAG - 3'

SGS1.2ΔV518

F: 5' - GCACAACCAGATAAAAGTGAATCAGAGTTAGTCAATCAAATAATAGAG - 3'

R: 5' - CTCTATTATTTGATTGACTAACTCTGATTCACTTTTATCTGGTTGTGC - 3'

SGS1.2ΔV518

F: 5' - AGCAGTTAATAAAAAAGGAAAAGGTCTATCTGGCATGGGTACCAG - 3'

R: 5' - CTGGTACCCATGCCAGATAGACCTTTTCCTTTTTTATTAAGTCT - 3'

B.1.3 Substrate Template/Primer for Biochemical Analysis:

All T/Ps were annealed and labeled as described in section 4.4 Material and Methods.

P19: 3' -GGAAACCTACCACGATGTT-5'

T36: 5' -AGAGCCCCCGAGACCTTTGGATGGTGCTACAAGCT-3'

P20: 3' -AGAGATCGTCACCGCGGGCT-5'

T36: 5' -CTCAGACCCTTTTAGTCAGAATGGAAAATCTCTAGCAGTGGCGCCCGAACAG-3'

P23: 3' -CTCTGGAAACCTACCACGATGTT-5'

T36: 5' -CTCAGACCCTTTTAGTCAGAATGGAAAATCTCTAGCAGTGGCGCCCGAACAG-3'

P23[◆]: 3' -CdTdCdTdGdGdAdAdAdCdCdTdAdCdCdAdCdGdAdTdGdTdT-5'

T36[✧]: 5' -rCrArGrArGrCrCrCrCrCrGrArGrArCrCrUrUrUrGrGrArUrGrGrUrGrCrUrArCrArArGrCrU-3'

◆DNA sequence

✧RNA sequence

B.1.4 Sequencing Primers (all forward):

P6RT is a forward direction primer and is designed to specifically anneal 5'-upstream the RT cloning site within the expression and cloning plasmid p6HRT_{xxLAI-MO},

P6RT

5' -GATCCCAGCTTCCCATT-3'

All primers listed below sequence in the forward direction and are designed to specifically anneal within the RT gene of the HIV-1_{xxLAI-MO} sequence, Appendix A.

RT-2066(+)

5' -GGACCTACACCTGTCAAC-3'

RT-S2

5' -GTTCCCTTAGATGAAGAC-3'

RT+6

5' -GAGGAACCAAAGCACTAA-3'

RT+8

5' -CACCCCTAACTGACACAAC-3'

B.1.5 Cloning Primers:

The primers described below are designed to bind and add restriction sites in the RT HIV-1_{xxLAI-MO} sequence, Appendix A [374].

3D 1989Bcl(+)

5' -GTTTTATCAAAGTAAGACAGTATGATCAGATAC-3'

3D BstBI(+)

5' -GTATTATGACCCTTCGAAAGACTTAATAG-3'

3D BstBI(-)

5' -CTATTAAGTCTTTCGAAGGGTCATAATAC-3'

3D HpaI(+)

5' -GAGTTTGTTAACACCCCTCCTTTAGT-3'

3D HpaI(-)

5' -GAGGGGTGTTAACAAACTCCCAC-3'

3D SgrAI(-)

5' -CTACCACCGGTGGCAGGTTA-3'

4232(-)

5' -CCTTGACTTTGGGGATTGTAGGGAA-3'

Bcl(+)

5' -AGGAAGATGGAAACCAAAAATGATAG-3'

The primers described below are designed to specifically anneal to sites in the PRO and IN genes of the HIV-1_{xxLAI-MO} sequence for amplification of full-length RT.

PRO-for

5' -GCTCTATTAGATACAGGAGCAGATGAT-3'

IN-rev

5' -CCTTCTAAATGTGTACAATCTAGTTGCCAT-3'

RT3908(-)

5' -CACAGCTGGCTACTATTTCTTTTG-3'

Whole RT-U

5' -AAGCTATAGGTACAGTATTAGTAGGACCTAC-3'

Whole RT-L

5' -TGCTCTCCAATTACTGTGATATTTCTCA-3'

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